Title: Mutant Viruses

Field of the Invention

5　The present invention relates to materials and methods
relating to the squamous cell carcinoma related oncogene
(SCCRO) and to mutant herpes simplex viruses.

Background to the Invention

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The herpes simplex virus (HSV) genome comprises two
covalently linked segments, designated long (L) and short
(S). Each segment contains a unique sequence flanked by a
pair of inverted terminal repeat sequences. The long
15　repeat (RL or R_L) and the short repeat (RS or R_S) are
distinct.

The HSV ICP34.5 (also γ34.5) gene, which has been
extensively studied[1,6,7,8], has been sequenced in HSV-1
20　strains F[9] and syn17+[3] and in HSV-2 strain HG52[4]. One copy
of the ICP34.5 gene is located within each of the RL
repeat regions. Mutants inactivating both copies of the
ICP34.5 gene (i.e. null mutants), e.g. HSV-1 strain 17
mutant 1716[2] (HSV1716) or the mutants R3616 or R4009 in
25　strain F[5], are known to lack neurovirulence, i.e. be
avirulent, and have utility as both gene delivery vectors
or in the treatment of tumours by oncolysis. HSV-1 strain
17 mutant 1716 has a 759bp deletion in each copy of the
ICP34.5 gene located within the BamHI s restriction
30　fragment of each RL repeat.

ICP34.5 null mutants such as 1716 are, in effect, first-generation oncolytic viruses. Most tumours exhibit individual characteristics and the ability of a broad spectrum first generation oncolytic virus to replicate in
5   or provide an effective treatment for all tumour types is not guaranteed.

HSV 1716 is described in EP 0571410 and WO 92/13943 and has been deposited on 28 January 1992 at the European
10   Collection of Animal Cell Cultures, Vaccine Research and Production Laboratories, Public Health Laboratory Services, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number V92012803 in accordance with the provisions of the Budapest Treaty on
15   the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (herein referred to as the 'Budapest Treaty').

Squamous cell carcinoma of the head and neck afflicts an
20   estimated 125,000 patients annually in Europe, North America and the Far East. Primary therapy for localized disease is surgery and adjuvant radiotherapy. Tumours recur in approximately one-third of patients. Once the cancer has recurred and/or metastasized, the patient is
25   considered incurable. Combination chemotherapy induces responses in 30-50% of patients but there is no clear impact on survival. There remains an urgent need for more effective therapies [12,13].

30   There has been much interest in the use of novel therapies in this disease with particular focus on oncolytic viruses by direct intratumoural injection. The

use of oncolytic viruses to selectively kill tumours while leaving normal cells unaffected is a very attractive concept as it has the potential to limit the toxicity which occurs with conventional modalities.

5   Recent research has been carried out using intratumoural injections of a selectively replicating adenovirus (Onyx-015) for the local control of recurrent disease. Phase I/II studies involving virus alone and in combination with chemotherapy have produced encouraging results

10   [14,15,16]

Selectively replicating Herpes simplex viruses HSV may have better efficacy due to its more potent replication and oncolytic potential. HSV1716[17] is a deletion mutant

15   of herpes simplex virus which fails to synthesise the virulence protein ICP34.5. It has been shown that HSV1716 replicates in actively dividing cells but not in resting or terminally differentiated cells[18,19]. In vivo, HSV1716 administration has been carried out in mouse

20   models of a range of cancers including melanoma, teratocarcinoma, glioma, medulloblastoma and mesothelioma. Animals showed improved survival and tumour regression following administration of HSV1716 [20,21,22,23,24,25] with no evidence of replication in normal

25   tissue and no toxicity. HSV1716 has been used in Phase 1 trials in patients with glioblastoma multeforme (GBM)[26], melanoma and head and neck cancer. No toxicity has been experienced and patients who were seropositive pre HSV1716 seroconverted and evidence of virus replication

30   contained within tumours has been obtained.

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It has been shown that the novel oncogene SCCRO (Squamous
cell carcinoma related oncogene (also called Oncoseq and
sometimes called SCRO)) is amplified in 30% of mucosal
squamous cell cancers and that overexpression is
5   associated with poor prognosis in head and neck cancer
patients.

The Oncoseq nucleic acid sequence was described in US
10/361,725 having publication number US 2004/0009541,
10   published on 15 January 2004. This document is
incorporated herein in its entirety by reference. A
polynucleotide sequence including an open reading frame
of 780 nucleotides for Oncoseq and the amino acid
sequence of the 259-residue polypeptide encoded thereby
15   was reported.

US 2004/0009541 describes Oncoseq alleles to be oncogenes
identified in primary squamous cell carcinoma tissues as
being colocalised with the highest gene duplication peak
20   within the 3q26.3 locus using a positional cloning
approach with Oncoseq being highly duplicated in those
carcinomas. Overexpression of Oncoseq is described to be
correlated with gene duplication, aggressive clinical
behaviour and malignant transformation in vitro, making
25   it a strong candidate as the target for 3q amplification.
The gene is described to be highly oncogenic and to have
a basic region-helix-loop-helix-leucine zipper motif,
suggesting it may function as a transcription factor.

30   RNAi

RNAi utilises small double-stranded RNA molecules (dsRNA) to target messenger RNA (mRNA), the precursor molecule that cells use to translate the genetic code into functional proteins. During the natural process of RNAi,

5   dsRNA is processed into short-interfering RNA (siRNA) duplexes of 21 nucleotides in length, and it is these molecules which recognise and target homologous (endogenous) mRNA sequences for enzymatic degradation (by complementary base-pair binding), resulting in gene

10   silencing.

The advantages of RNAi over other gene-targeting strategies such as anti-sense oligonucleotides include its relative specificity, its enhanced efficacy (only

15   nanomolar quantities of siRNA are required for efficient gene-silencing), and the fact that siRNA treatment feeds into a natural RNAi pathway that is inherent to all cells.

20   The success of gene-silencing by siRNA can be highly variable depending on the gene target and cell type being targeted.

Summary of the Invention

25

The inventors have used plasmid RL1.dIRES-GFP to generate a shuttle vector, designated RL1.dCMV-asSCCRO-GFP, containing the human antisense squamous cell carcinoma related oncogene (SCCRO) arranged in an orientation

30   downstream of a CMV IE promoter to produce antisense RNA transcripts for use in antisense therapeutic methods.

6

Using this shuttle vector the inventors have provided a
novel second generation mutant HSV, designated
HSV1716/CMV-asSCCRO/GFP (also called HSV1716asSCCRO).
The genome of this mutant HSV comprises the nucleic acid
5   encoding heterologous (i.e. non-HSV originating)
antisense SCCRO inserted at one or each ICP34.5 locus,
disrupting the ICP34.5 protein coding sequence such that
the ICP34.5 gene is non-functional and cannot express a
functional ICP34.5 gene product. The generated HSV is
10   capable of expressing an antisense RNA transcript under
control of the CMV IE promoter which is capable of
inhibiting the action of the SCCRO gene by binding to
sense SCCRO nucleotide sequences, e.g. SCCRO mRNA or
genomic SCCRO. This virus retains the oncolytic activity
15   of HSV-1 strain 17 mutant 1716 and can be used in
targeted antisense nucleotide delivery strategies and
therapeutic methods.

In an alternative arrangement, instead of integrating a
20   nucleic acid encoding an antisense, the inventors have
integrated an siRNA in the genome of a herpes simplex
virus. This siRNA is preferably heterologous to the
herpes simplex virus and may be expressed from the herpes
simplex virus genome. In one preferred embodiment the
25   integrated nucleic acid encodes an siRNA capable of
targeting and repressing or inhibiting expression of the
functional SCCRO gene product. When expressed, the siRNA
operates to silence, wholly or in part, expression of the
functional SCCRO gene product.
30

The heterologous asSCCRO expressed by an herpes simplex
virus according to the present invention may be useful in

RNA based antisense therapeutic techniques for repression
or silencing of the SCCRO gene product or of it's
expressed function.

5   The siRNA expressed by an herpes simplex virus according
to the present invention may be useful in siRNA based
therapeutic techniques for tissue specific repression or
silencing of the SCCRO gene product or of it's expressed
function.
10

At its most general the present invention relates to (i)
materials and methods relating to the squamous cell
carcinoma related oncogene; and (ii) mutant herpes
simplex viruses.
15

In one embodiment of the present invention, there is
provided an attenuated replication competent HSV
expressing antisense SCCRO, namely, HSV1716asSCCRO, which
may be used in the treatment of squamous cell cancer,
20   particularly head and neck squamous cell cancer.

Accordingly the present invention further provides a
pharmaceutical composition comprising HSV1716asSCCRO and
the use of such virus and/or composition in the treatment
25   of cancer.

According to one aspect of the present invention there is
provided an herpes simplex virus wherein the herpes
simplex virus genome comprises nucleic acid encoding an
30   antisense to the squamous cell carcinoma related oncogene
(asSCCRO).

Said nucleic acid may encode an antisense to a mammalian
squamous cell carcinoma related oncogene, more preferably
an antisense to a human squamous cell carcinoma related
oncogene.

In one arrangement said nucleic acid may encode a
nucleotide sequence complementary to:

  (i)   the polynucleotide sequence of SEQ ID No.s 1 or
          3 or its complement;
  (ii)   the mRNA transcript of SEQ ID No.s 1 or 3; or
  (iii)   to a fragment of said polynucleotide sequence,
          complement or mRNA transcript.

In another arrangement said nucleic acid may encode a
nucleotide sequence having at least 60% sequence identity
to the nucleotide sequence complementary to:

  (i)   the polynucleotide sequence of SEQ ID No.s 1 or
          3 or its complement;
  (ii)   the mRNA transcript of SEQ ID No.s 1 or 3; or
  (iii)   to a fragment of said polynucleotide sequence
          or mRNA transcript.

More preferably said degree of sequence identity may be
at least 70%, 80%, 90%, 95%, 96%, 97%, 98% or 99%. The
fragment referred to at (iii) may comprise at least 20
nucleotides and may be limited to no more than 900
nucleotides. Identity of sequences is determined across
the entire length of a given nucleotide sequence. Where
sequences are of different length, sequence identity of
the shorter sequence is determined over the entire length
of the longer sequence.

In another arrangement said nucleic acid may be selected
as one that hybridises to:

(i)   the polynucleotide sequence of SEQ ID No.s 1 or
        3 or its complement;

5   (ii)   the mRNA transcript of SEQ ID No.s 1 or 3; or

(iii)   to a fragment of said polynucleotide sequence
        or mRNA transcript

under high or very high stringency conditions.

10   The genome of Herpes simplex viruses according to the
present invention may further comprises a regulatory
sequence operably linked to said nucleic acid encoding an
antisense to the squamous cell carcinoma related
oncogene, wherein said regulatory sequence has a role in
15   controlling transcription of said siRNA.

In a further aspect of the present invention there is
provided an herpes simplex virus wherein the herpes
simplex virus genome comprises nucleic acid encoding a
20   short interfering ribonucleic acid (siRNA) molecule that
is capable of repressing or silencing expression of the
squamous cell carcinoma related oncogene (SCCRO) nucleic
acid or polypeptide.

25   Said siRNA may repress or silence expression of a
mammalian SCCRO, more preferably of a human SCCRO.

Said nucleic acid encoding siRNA may comprise a nucleic
acid of between 10 and 50 nucleotides in length and may
30   have the sequence of SEQ ID No.5 or the complement
thereof.

In another arrangement said nucleic acid encoding siRNA
may comprise a nucleic acid of between 10 and 50
nucleotides in length and may have at least 70%, 80%,
90%, 95%, 96%, 97%, 98% or 99% identity to SEQ ID No.5 or
5   the complement thereof. Identity of sequences is
determined across the entire length of a given nucleotide
sequence. Where sequences are of different length,
sequence identity of the shorter sequence is determined
over the entire length of the longer sequence.

10

In another arrangement said nucleic acid encoding siRNA
may be selected as one that hybridises to the nucleic
acid of SEQ ID No. 5 or its complement under high or very
high stringency conditions.

15

The genome of said herpes simplex virus may further
comprise a regulatory sequence operably linked to said
siRNA, wherein said regulatory sequence has a role in
controlling transcription of said siRNA.

20

The nucleic acid encoding asSCCRO or said siRNA may be
located in at least one RL1 locus of the herpes simplex
virus genome. Suitably it may be located in, or overlap,
at least one of the ICP34.5 protein coding sequences of
25   the herpes simplex virus genome. The nucleic acid may be
located in both (usually this is all) copies of the RL1
locus or ICP34.5 protein coding sequence.

The herpes simplex virus is preferably a mutant and may
30   be a mutant of HSV-1 or HSV-2, more preferably of one of
HSV-1 strains 17, F or HSV-2 strain HG52. The herpes

simplex virus may be a further mutant of HSV-1 strain 17
mutant 1716.

In certain arrangements the herpes simplex virus may be a
gene specific null mutant, such as an ICP34.5 null
mutant.

In other arrangements the herpes simplex virus may lack
at least one expressible ICP34.5 gene.

In yet another arrangement the herpes simplex virus may
lack only one expressible ICP34.5 gene.

In yet another arrangement the herpes simplex virus may
be non-neurovirulent.

In herpes simplex viruses of the present invention the
nucleic acid encoding the asSCCRO or said siRNA may form
part of a nucleic acid cassette permanently integrated in
the herpes simplex virus genome, said cassette comprising
nucleic acid encoding :

      (a)   said asSCCRO or said siRNA; and nucleic
            acid encoding:

      (b)   a ribosome binding site or a first
            regulatory nucleotide sequence; and

      (c)   a marker,

wherein the nucleic acid encoding said asSCCRO or siRNA
is arranged upstream (5´) of the ribosome binding site or
first regulatory nucleotide sequence and the ribosome
binding site or first regulatory nucleotide sequence is
arranged upstream (5´) of the marker. Said first

regulatory sequence may have a role in controlling
transcription of said marker.

A second regulatory nucleotide sequence may be located
upstream (5') of the nucleic acid encoding asSCCRO or said
siRNA, wherein the regulatory nucleotide sequence has a
role in controlling and regulating transcription of the
nucleic acid encoding the asSCCRO or siRNA and hence
expression of the resulting transcript. The regulatory
sequences may comprise selected promoter or enhancer
elements known to the person skilled in the art, e.g. the
CytoMegalovirus (CMV) or phosphoglycerokinase (PGK)
promoters.

The components of the cassette are preferably arranged in
a predetermined order.

In one preferred arrangement, the nucleic acid encoding
the asSCCRO is arranged upstream (i.e. 5') of the
ribosome binding site and the ribosome binding site is
arranged upstream (i.e. 5') of the marker. During
transcription a single transcript may be produced from
the cassette comprising a first cistron comprising the
asSCCRO and a second cistron encoding the marker wherein
the ribosome binding site is located between the
cistrons.

A transcription product of this cassette may be a bi- or
poly- cistronic transcript comprising a first cistron
encoded by the nucleic acid encoding the asSCCRO and a
second cistron encoding the marker nucleic acid wherein

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the ribosome binding site is located between said first
and second cistrons.

5   In another preferred arrangement, the nucleic acid
encoding the siRNA is arranged upstream (i.e. 5') of a
first regulatory nucleotide sequence and the first
regulatory nucleotide sequence is arranged upstream (i.e.
5') of the marker.

10   The cassette may disrupt a protein coding sequence of the
herpes simplex virus genome resulting in inactivation of
the respective gene product.

Nucleic acid encoding a selected antisense DNA, that is
15   DNA corresponding to a gene component (e.g. regulatory
sequence, 5' UTR, 3'UTR or protein coding sequence) or
fragment of a gene component, is inserted in the cassette
in an orientation such that upon transcription an
antisense RNA is obtained. Thus the expressed product of
20   the cassette may ultimately be an antisense nucleic acid,
preferably RNA.

One suitable ribosome binding site comprises a ribosome
entry site permitting entry of a ribosome to the
25   transcribed mRNA encoded by the nucleic acid of the
cassette such that the ribosome binds to the translation
start signal. Preferably, the ribosome entry site is an
internal ribosome entry site (IRES), more preferably an
encephalomyocarditis virus IRES, permitting cap-
30   independent initiation of translation. The IRES thus
enables translation of a coding sequence located
internally of a bi- or poly- cistronic mRNA, i.e. of a

cistron located downstream of an adjacent cistron on a single transcript.

Preferably the marker is a defined nucleotide sequence coding for a polypeptide which can be expressed in a cell line (e.g. BHK cells) infected with mutant herpes simplex virus into which the cassette has been recombined. The function of the marker is to enable identification of virus plaques containing mutant virus transformed with the cassette.

The marker is preferably a detectable marker, more preferably an expressible marker polypeptide or protein comprising at least the coding sequence for the selected polypeptide or protein. The nucleic acid encoding the marker may further comprise regulatory sequence upstream and/or downstream of the coding sequence having a role in control of transcription of the marker mRNA. Preferred markers include the Green Fluorescent Protein (GFP) protein coding sequence or gene, preferably the enhanced Green Fluorescent Protein (EGFP) protein coding sequence or gene.

In other arrangements the marker may comprise a defined nucleotide sequence which can be detected by hybridisation under high stringency conditions with a corresponding labelled nucleic acid probe, e.g. using a fluorescent- or radio-label.

The cassette may also comprise nucleic acid encoding a polyadenylation ("polyA") sequence, which sequence is preferably located downstream (3´) of the nucleic acid

encoding the marker. One preferred polyA sequence is the
Simian Virus 40 (SV40) polyadenylation sequence. The
preferred location of the polyA sequence within the
cassette is immediately downstream (i.e. 3') of the
5   marker.

By antisense nucleic acid is meant a nucleic acid:

(i)   having substantial sequence identity to the
        nucleic acid formed by the sequence of
10   complementary bases to the single strand of a
        target nucleic acid; and/or

(ii)   a nucleic acid which hybridises to the target
        nucleic acid under intermediate, high or very
        high stringency conditions.

15

In accordance with aspects of the present invention, the
target nucleic acid may be an SCCRO polynucleotide
sequence (e.g. gene sequence), the polynucleotide coding
sequence for the SCCRO polypeptide or protein, or a part/
20   fragment of the gene or polypeptide coding sequence.
Thus, the antisense nucleic acid may be useful in binding
the target nucleic acid (e.g. the SCCRO genomic coding
sequence or mRNA transcript) and may be used as an
inhibitor to prevent or disrupt the normal expression,
25   activity, folding or binding of the target nucleic acid.
The substantial sequence identity is preferably at least
50% sequence identity, more preferably one of at least
60, 70, 75, 80, 85, 90, 92, 94, 95, 96, 97, 98, 99 or
100% identity. Identity of sequences is determined across
30   the entire length of a given nucleotide sequence. Where
sequences are of different length, sequence identity of

the shorter sequence is determined over the entire length
of the longer sequence.

The antisense nucleic acid may comprise all or a fragment
of the antisense to the squamous cell carcinoma related
oncogene (asSCCRO), preferably it is an antisense to the
human SCCRO.

The nucleic acid encoding the asSCCRO which may form part
of the inserted cassette may encode a full length
transcript of the antisense nucleotide sequence to the
SCCRO. That full length antisense transcript may be a
sequence complementary to one of the polynucleotide
sequences of SEQ ID No.1 or SEQ ID No.3 or their
complementary sequences. Alternatively, the nucleic acid
may encode one or more fragments of the full length
antisense transcript.

A fragment may comprise a nucleotide sequence encoding at
least 10% of the corresponding full length sequence, more
preferably the fragment comprises at least 20, 30, 40,
50, 60, 70, 80, 85, 90, 95, 96, 97, 98 or 99% of the
corresponding full length sequence. Preferably, the
fragment comprises at least, i.e. has a minimum length
of, 20 nucleotides, more preferably at least 30, 40, 50,
100, 150, 200, 300, 400, 500, 600, 700, 800, 900, 1000,
1100, 1200, 1300, 1400, 1500, 1600, 1700, 1800, 1900
2000, 2100, 2200, 2300, 2400, 2500, 2600, 2700, 2800,
2900, 3000, 3100, 3200, 3300, 3400, 3500, 3600, 3700,
3800, 3900 or 4000 nucleotides. The fragment may have a
maximum length, i.e. be no longer than, 20 nucleotides,
more preferably no longer than 30, 40, 50, 100, 150, 200,

300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200,
1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100,
2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000,
3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or
4000. The fragment length may be anywhere between said
minimum and maximum length.

In the case of an antisense to the SCCRO, a full length
transcript comprises a minimum of the contiguous sequence
of nucleotides forming an antisense strand to the
corresponding complete nucleotide sequence encoding the
full amino acid sequence of the SCCRO gene product or to
is compliment. The complete nucleotide sequence of the
SCCRO gene product may comprise the region of SEQ ID No.1
or SEQ ID No.3 respectively encoding the polypeptide of
SEQ ID No. 2 or SEQ ID No.4.

Preferred antisense nucleic acids may single stranded and
may be DNA or RNA.

Preferred siRNA may be single or double stranded and may
comprise single stranded nucleic acids capable of forming
duplex structures by stem-loop formation and self-binding
of complementary nucleotides. Preferred siRNA may include
RNA molecules having a sequence encoded by SEQ ID No. 5
or its complement and nucleic acids having a sequence
identity of at least 60% to SEQ ID No. 5 or a
complementary sequence thereof, and more preferably
having at least 70, 80, 85, 90, 95% or 100% sequence
identity. Identity of sequences is determined across the
entire length of a given nucleotide sequence. Where
sequences are of different length, sequence identity of

the shorter sequence is determined over the entire length
of the longer sequence.

Preferred siRNA or nucleic acid encoding preferred siRNA
5   may comprise nucleotide sequences heterologous to the
selected HSV strain being modified, i.e. the siRNA or
nucleic acid sequence encoding the siRNA does not occur
in or originate from the parental, unmodified wild-type,
virus.
10

Furthermore Herpes simplex viruses according to aspects
of the present invention may contain nucleic acid,
encoding siRNA molecules, which hybridise with SEQ ID No
5 or its complement under very high, high or intermediate
15   stringency conditions.

siRNA molecules encoded by nucleic acid molecules
integrated in the genome of Herpes simplex viruses
according to the present invention may be of any length,
20   but preferred siRNA molecules are small and may comprise
at least 10 nucleotides and no more than 50 nucleotides.
Herpes simplex virus according to the present invention
may encode an siRNA which is a fragment of the siRNA
encoded by SEQ ID No.5. Particularly suitable siRNA will
25   have a single strand length in the range 10 to 30
nucleotides and more suitably in the range 15 to 25
nucleotides.   Selected siRNA molecules may be any of 15,
16, 17, 18, 19, 20, 21, 22, 23, 24 or 25 nucleotides in
length. SiRNA molecules which fold to form their own
30   duplex structures, e.g. by stem-loop formation will thus
have an unfolded single strand length of about two times

the number or range recited above and siRNA and nucleic
acid encoding such siRNA may be particularly preferred.

Herpes simplex viruses according to aspects of the
5   present invention may contain nucleic acid encoding siRNA
molecules having one or more of these sequences.

Mutant herpes simplex viruses of the present invention
may be generated by site directed insertion of a nucleic
10 acid cassette into the viral genome, more preferably by
homologous recombination. However, the viruses of the
invention are not limited to Herpes simplex viruses
obtained in this way.

15 In other aspects of the present invention herpes simplex
viruses according to the present invention are provided
for use in a method of medical treatment. Suitably they
are provided for use in the treatment of disease.
Preferably they are provided for use in the treatment of
20 cancer. Suitably they may be provided for use in the
oncolytic treatment of cancer/a tumour. The use of
herpes simplex viruses according to the present invention
in the manufacture of a medicament for the treatment of
cancer is also provided.
25

In another aspect of the present invention medicaments
comprising herpes simplex virus mutants according to the
present invention for use in oncotherapy and methods of
treating tumours comprising administering to a patient in
30 need of treatment an effective amount of a mutant HSV or
a medicament comprising or derived from such HSV are also
provided. Methods of lysing or killing tumour cells in

vitro or in vivo comprising the step of administering to
a patient in need of treatment an amount of an Herpes
simplex virus according to the present invention are also
provided.

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A medicament, pharmaceutical composition or vaccine
comprising an Herpes simplex virus according to the
present invention is also provided. The medicament,
pharmaceutical composition or vaccine may further

10 comprise a pharmaceutically acceptable carrier, adjuvant
or diluent.

The present invention may also include the following
aspects which may be provided in combination with any of

15 the other aspects and features described.

In another aspect of the present invention there is
provided an herpes simplex virus, wherein the genome of
said virus comprises a nucleic acid sequence encoding an

20 antisense to the squamous cell carcinoma related oncogene
(asSCCRO) in at least one of the long repeat regions (R_L).

In another aspect of the present invention there is
provided an herpes simplex virus, wherein the genome of

25 said virus comprises a nucleic acid sequence encoding an
antisense to the squamous cell carcinoma related oncogene
(asSCCRO) and wherein the herpes simplex virus is non-
neurovirulent.

30 In another aspect of the present invention there is
provided an herpes simplex virus for use in the treatment
of a tumour, wherein the genome of said virus comprises a

nucleic acid sequence encoding an antisense to the squamous cell carcinoma related oncogene (asSCCRO) in at least one of the long repeat regions (R_L).

5   In another aspect of the present invention there is provided an herpes simplex virus for use in the treatment of a tumour, wherein the genome of said virus comprises a nucleic acid sequence encoding an antisense to the squamous cell carcinoma related oncogene (asSCCRO) and
10   wherein the herpes simplex virus is non-neurovirulent.

In another aspect of the present invention the use of an herpes simplex virus, wherein the genome of said virus comprises a nucleic acid sequence encoding an antisense
15   to the squamous cell carcinoma related oncogene (asSCCRO) in at least one of the long repeat regions (R_L), in the manufacture of a medicament for the treatment of cancer is provided.

20   In another aspect of the present invention the use of an herpes simplex virus, wherein the genome of said virus comprises a nucleic acid sequence encoding an antisense to the squamous cell carcinoma related oncogene (asSCCRO) and wherein the herpes simplex virus is non-
25   neurovirulent, in the manufacture of a medicament for the treatment of cancer is provided.

In another aspect of the present invention there is provided a method for the treatment of a tumour
30   comprising the step of administering to a patient in need of treatment an effective amount of an herpes simplex virus, wherein the genome of said virus comprises a

nucleic acid sequence encoding an antisense to the
squamous cell carcinoma related oncogene (asSCCRO) in at
least one of the long repeat regions (R_L).

5   In another aspect of the present invention there is
provided a method for the treatment of a tumour
comprising the step of administering to a patient in need
of treatment an effective amount of an herpes simplex
virus, wherein the genome of said virus comprises a
10   nucleic acid sequence encoding an antisense to the
squamous cell carcinoma related oncogene (asSCCRO) and
wherein the herpes simplex virus is non-neurovirulent.

Suitably, in the methods of treatment of a tumour the
15   herpes simplex virus is capable of killing tumour cells.

In another aspect of the present invention there is
provided a method of expressing in vitro or in vivo an
antisense to the squamous cell carcinoma related oncogene
20   (asSCCRO), said method comprising the step of infecting
at least one cell or tissue of interest with a herpes
simplex virus, wherein the genome of said virus comprises
a nucleic acid sequence encoding asSCCRO in at least one
of the long repeat regions (R_L), said asSCCRO operably
25   linked to a transcription regulatory sequence.

In another aspect of the present invention there is
provided a method of expressing in vitro or in vivo an
antisense to the squamous cell carcinoma related oncogene
30   (asSCCRO), said method comprising the step of infecting
at least one cell or tissue of interest with a non-
neurovirulent herpes simplex virus, wherein the genome of

said virus comprises a nucleic acid sequence encoding
asSCCRO, said asSCCRO operably linked to a transcription
regulatory sequence.

5   In another aspect of the present invention there is
provided an herpes simplex virus, wherein the genome of
said virus comprises a nucleic acid sequence encoding a
short interfering ribonucleic acid (siRNA) molecule that
is capable of repressing or silencing expression of
10   squamous cell carcinoma related oncogene (SCCRO) nucleic
acid or polypeptide in at least one of the long repeat
regions (R_L).

In another aspect of the present invention there is
15   provided an herpes simplex virus, wherein the genome of
said virus comprises a nucleic acid sequence encoding a
short interfering ribonucleic acid (siRNA) molecule that
is capable of repressing or silencing expression of
squamous cell carcinoma related oncogene (SCCRO) nucleic
20   acid or polypeptide and wherein the herpes simplex virus
is non-neurovirulent.

In another aspect of the present invention there is
provided an herpes simplex virus for use in the treatment
25   of a tumour, wherein the genome of said virus comprises a
nucleic acid sequence encoding a short interfering
ribonucleic acid (siRNA) molecule that is capable of
repressing or silencing expression of squamous cell
carcinoma related oncogene (SCCRO) nucleic acid or
30   polypeptide in at least one of the long repeat regions
(R_L).

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In another aspect of the present invention there is provided an herpes simplex virus for use in the treatment of a tumour, wherein the genome of said virus comprises a nucleic acid sequence encoding a short interfering

5   ribonucleic acid (siRNA) molecule that is capable of repressing or silencing expression of squamous cell carcinoma related oncogene (SCCRO) nucleic acid or polypeptide and wherein the herpes simplex virus is non-neurovirulent.

10

In another aspect of the present invention the use of an herpes simplex virus, wherein the genome of said virus comprises a nucleic acid sequence encoding a short interfering ribonucleic acid (siRNA) molecule that is

15   capable of repressing or silencing expression of squamous cell carcinoma related oncogene (SCCRO) nucleic acid or polypeptide in at least one of the long repeat regions (R_L), in the manufacture of a medicament for the treatment of cancer is provided.

20

In another aspect of the present invention the use of an herpes simplex virus, wherein the genome of said virus comprises a nucleic acid sequence encoding a short interfering ribonucleic acid (siRNA) molecule that is

25   capable of repressing or silencing expression of squamous cell carcinoma related oncogene (SCCRO) nucleic acid or polypeptide and wherein the herpes simplex virus is non-neurovirulent, in the manufacture of a medicament for the treatment of cancer is provided.

30

In another aspect of the present invention there is provided a method for the treatment of a tumour

comprising the step of administering to a patient in need
of treatment an effective amount of an herpes simplex
virus, wherein the genome of said virus comprises, in at
least one of the long repeat regions (R_L), a nucleic acid

5   sequence encoding a short interfering ribonucleic acid
(siRNA) molecule that is capable of repressing or
silencing expression of squamous cell carcinoma related
oncogene (SCCRO) nucleic acid or polypeptide.

10   In another aspect of the present invention there is
provided a method for the treatment of a tumour
comprising the step of administering to a patient in need
of treatment an effective amount of an herpes simplex
virus, wherein the genome of said virus comprises a

15   nucleic acid sequence encoding a short interfering
ribonucleic acid (siRNA) molecule that is capable of
repressing or silencing expression of squamous cell
carcinoma related oncogene (SCCRO) nucleic acid or
polypeptide and wherein the herpes simplex virus is non-

20   neurovirulent.

Suitably, in the methods of treatment of a tumour the
herpes simplex virus is capable of killing tumour cells.

25   In another aspect of the present invention there is
provided a method of expressing in vitro or in vivo a
short interfering ribonucleic acid (siRNA) molecule that
is capable of repressing or silencing expression of
squamous cell carcinoma related oncogene (SCCRO) nucleic

30   acid or polypeptide, said method comprising the step of
infecting at least one cell or tissue of interest with a
herpes simplex virus, wherein the genome of said virus

comprises a nucleic acid sequence encoding said siRNA in
at least one of the long repeat regions (R_L), wherein said
nucleic acid sequence encoding said siRNA is operably
linked to a transcription regulatory sequence.

5

In another aspect of the present invention there is
provided a method of expressing in vitro or in vivo a
short interfering ribonucleic acid (siRNA) molecule that
is capable of repressing or silencing expression of

10   squamous cell carcinoma related oncogene (SCCRO) nucleic
acid or polypeptide, said method comprising the step of
infecting at least one cell or tissue of interest with a
non-neurovirulent herpes simplex virus, wherein the
genome of said virus comprises a nucleic acid sequence

15   encoding said siRNA, wherein said nucleic acid sequence
encoding said siRNA is operably linked to a transcription
regulatory sequence.

siRNA according to the invention preferably repress the

20   function of the squamous cell carcinoma related oncogene
(SCCRO) protein.

In another aspect of the present invention a method is
provided for repressing the cellular expression of the

25   squamous cell carcinoma related oncogene (SCCRO) in vitro
comprising the step of: in vitro, contacting a cell with
an herpes simplex virus of the present invention or
pharmaceutical composition containing such virus.

30   In one preferred aspect of the invention the herpes
simplex virus is HSV1716/CMV-asSCCRO/GFP, deposited as
'HSV1716asSCCRO', in the name of Crusade Laboratories

Limited having an address at Department of Neurology
Southern General Hospital 1345 Govan Road Govan Glasgow
G51 5TF Scotland on 19 May 2004 at the European
Collection of Cell Cultures (ECACC), Health Protection
5   Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG,
United Kingdom under accession number 04051901 in
accordance with the provisions of the Budapest Treaty on
the International Recognition of the Deposit of
Microorganisms for the Purposes of Patent Procedure
10   (herein referred to as the 'Budapest Treaty').

In yet another aspect of the present invention a cell, in
vitro, in which expression of the squamous cell carcinoma
related oncogene (SCCRO) protein or nucleic acid is
15   repressed or silenced is provided. The cell may be a
mammalian cell, preferably a human cell.

Suitably, the administration of said herpes simplex virus
may comprise parenteral administration. Preferably
20   administration of the herpes simplex virus is by
injection, more preferably injection to the tumour which
is to be treated. Alternatively injections may be
intravenous.

25   In a further aspect of the present invention in vitro or
in vivo methods are provided for delivery of nucleic acid
encoding asSCCRO or siRNA to at least one cell or to a
tissue of interest said method comprising the step of
infecting said cell(s) or tissue with a herpes simplex
30   virus according to the invention.

In another aspect of the present invention a method of
making or producing a modified herpes simplex virus of
the invention is provided comprising the step of
introducing a nucleic acid sequence encoding asSCCRO or

5   siRNA at a selected or predetermined insertion site in
the genome of a selected herpes simplex virus.

As described, the nucleic acid sequence encoding the
asSCCRO or siRNA may form part of a nucleic acid cassette

10   which is inserted in the genome of a selected herpes
simplex virus by homologous recombination. Whether part
of a cassette or not, the site of insertion may be in any
genomic location selected. One preferred insertion site
is in one or both of the long repeat regions (R_L), and one

15   copy of the cassette is preferably inserted in each copy
of the long repeat (R_L). More preferably the insertion
site is in at least one (preferably both) RL1 locus and
most preferably it is inserted in at least one
(preferably both) of the ICP34.5 protein coding sequences

20   of the HSV genomic DNA. It is preferred that the
insertion occurs in identical or substantially similar
positions in each of the two repeat regions, RL1 loci or
ICP34.5 protein coding sequences.

25   Insertion may be such as to produce a modified virus
which is a non-neurovirulent mutant capable of expressing
the encoded asSCCRO or siRNA upon transfection into
mammalian, more preferably human, cells in vivo and in
vitro. The non-neurovirulent mutant may be an ICP34.5

30   null mutant.

The nucleic acid cassette may be of any size, e.g. up to 5, 10, 15, 20, 25, 30, 35, 40, 45 or 50Kbp in length.

Preferably, the herpes simplex virus contains at least
5 one copy of the nucleic acid encoding the asSCCRO or siRNA in each long repeat region (R_L), i.e. in the terminal and internal long repeat (TR_L and IR_L) regions. In a preferred arrangement each exogenous sequence or cassette is located in an RL1 locus of the herpes simplex
10 virus genome, more preferably in the DNA of the herpes simplex virus genome encoding the ICP34.5 gene or protein coding sequence. The herpes simplex virus thereby lacks neurovirulence.

15 The parent herpes simplex virus, from which a virus of the invention is derived may be of any kind, e.g. HSV-1 or HSV-2. In one preferred arrangement the herpes simplex virus is a variant of HSV-1 strain 17 and may be obtained by modification of the strain 17 genomic DNA.
20 Suitable modifications include the insertion of the exogenous asSCCRO or siRNA nucleic acid sequences or exogenous/ heterologous cassette comprising said sequence into the herpes simplex virus genomic DNA. The insertion may be performed by homologous recombination of the
25 exogenous nucleic acid sequence into the genome of the selected herpes simplex virus.

Although the non-neurovirulent phenotype of the herpes simplex virus of the invention may be the result of
30 insertion of the exogenous nucleic acid sequence in the RL1 locus, herpes simplex viruses according to the present invention may be obtained by utilising a non-

neurovirulent parent strain, e.g. HSV1716 deposited under
the Budapest Treaty at the European Collection of Animal
Cell Cultures (ECACC), Health Protection Agency, Porton
Down, Salisbury, Wiltshire, United Kingdom under

5   accession number V92012803, and inserting the exogenous
nucleic acid sequence at another location of the genome
by standard genetic engineering techniques, e.g.
homologous recombination. In this aspect the location of
the herpes simplex virus genome selected for insertion of

10 the asSCCRO or siRNA nucleic acid sequence or cassette
containing said sequence may be a neutral location.

Herpes simplex viruses of the present invention may be
variants of a known 'parent' strain from which the herpes

15 simplex virus of the invention has been derived. A
particularly preferred parent strain is HSV-1 strain 17.
Other parent strains may include HSV-1 strain F or HSV-2
strain HG52. A variant comprises an HSV in which the
genome substantially resembles that of the parent,

20 contains the asSCCRO or siRNA encoding nucleic acid
sequence or cassette containing said sequence and may
contain a limited number of other modifications, e.g.
one, two or three other specific mutations, which may be
introduced to disable the pathogenic properties of the

25 herpes simplex virus, for example a mutation in the
ribonucleotide reductase (RR) gene, the 65K trans
inducing factor (TIF) and/or a small number of mutations
resulting from natural variation, which may be
incorporated naturally during replication and selection

30 in vitro or in vivo. Otherwise the genome of the variant
will be that of the parent strain.

Herpes simplex viruses of the invention may be used in a method of medical treatment. This may involve treatment of diseases associated with or involving the proliferation of cells, or cancers or tumours of any

5 kind. Treatment may involve the selective lysis of dividing cells. This may be oncolysis, i.e. lysis of tumour cells. Tumours to be treated may be of any kind, may comprise cancers, neoplasms or neoplastic tissue and may be in any animal or human patient.

10

Herpes simplex viruses of the invention may be used in 'gene delivery' methods in vitro or in vivo. Non-neurovirulent herpes simplex viruses of the invention are expression vectors and may be used to infect selected

15 cells or tissues in order to express the asSCCRO or siRNA encoded by the herpes simplex virus genome.

In one arrangement, cells may be taken from a patient, a donor or from any other source, infected with a herpes

20 simplex virus of the invention, optionally screened for expression and/or function of the encoded asSCCRO or siRNA, and optionally returned/introduced to a patient's body, e.g. by injection.

25 Delivery of herpes simplex viruses of the invention to the selected cells may be performed using naked virus or by encapsulation of the virus in a carrier, e.g. nanoparticles, liposomes or other vesicles.

30 In vitro cultured cells, preferably human or mammalian cells, transformed with viruses of the present invention and preferably cells expressing the asSCCRO or siRNA as

well as methods of transforming such cells in vitro with
said viruses form further aspects of the present
invention.

5   Cancer/tumour types to be treated may include primary
     and/or secondary (metastatic) tumours. These may be
     carcinomas of the head and/or neck. They may be squamous
     cell carcinomas, which may be of mucosal origin and may
     show a predilection for duplication of the 3q locus.
10   Preferred squamous cell carcinomas to be treated may be
     those of the head and/or neck. Squamous cell carcinomas
     to be treated may include those originating from the
     lung, head neck, oesophagus and cervix.

15   Other tumour types which may be treated may be primary or
     secondary (metastatic) tumours. Tumours to be treated may
     be nervous or non-nervous system tumours. Nervous system
     tumours may originate either in the central or peripheral
     nervous system, e.g. glioma, medulloblastoma, meningioma,
20   neurofibroma, ependymoma, Schwannoma, neurofibrosarcoma,
     astrocytoma and oligodendroglioma. Non-nervous system
     tumours may originate in any other non-nervous tissue,
     examples include melanoma, mesothelioma, lymphoma,
     hepatoma, epidermoid carcinoma, prostate carcinoma,
25   breast cancer cells, lung cancer cells or colon cancer
     cells. HSV mutants of the present invention may be used
     to treat metastatic tumours of the central or peripheral
     nervous system which originated in a non-nervous system
     tissue.
30
     In this specification, a mutant herpes simplex virus is a
     non-wild type herpes simplex virus and may be a

recombinant herpes simplex virus. Mutant herpes simplex viruses may comprise a genome containing modifications relative to the wild type. A modification may include at least one deletion, insertion, addition or substitution.

5

Medicaments and pharmaceutical compositions according to aspects of the present invention may be formulated for administration by a number of routes, including but not limited to, parenteral, intravenous, intramuscular,

10   intratumoural, oral and nasal. The medicaments and compositions may be formulated in fluid or solid (e.g. tablet) form. Fluid formulations may be formulated for administration by injection to a selected region of the human or animal body.

15

In this specification, non-neurovirulence is defined by the ability to introduce a high titre of virus (approx 10^6 plaque forming units (pfu)) to an animal or patient[22, 23] without causing a lethal encephalitis such that the LD_{50}

20   in animals, e.g. mice, or human patients is in the approximate range of $\geq 10^6$ pfu[21].

Where all copies of the ICP34.5 gene present in the herpes simplex virus genome (two copies are normally

25   present) are disrupted such that the herpes simplex virus is incapable of producing a functional ICP34.5 gene product, the virus is considered to be an ICP34.5 null mutant.

30   A regulatory sequence (e.g. promoter) that is operably linked to a nucleotide sequence may be located adjacent to that sequence or in close proximity such that the

34

regulatory sequence can effect and/or control expression
of a product of the nucleotide sequence. The encoded
product of the nucleotide sequence may therefore be
expressible from that regulatory sequence.

5

SCCRO

The polynucleotide sequence of SEQ ID No.1, positions 43-
918 and the polynucleotide of SEQ ID No.2 are disclosed
10   in GenBank Accession No. AF456425 (GI:18700655) released
to the public as of 19 February 2002.

A second Oncoseq (Oncoseq2) polypeptide is encoded by the
polynucleotide sequence of SEQ ID No.3, which together
15   with the polypeptide thereby encoded (SEQ ID No.4) are
disclosed in GenBank Accession No. AF456426 (GI:18700657)
released to the public as of 19 February 2002.

The GenBank database may be accessed at
20   http://www.ncbi.nlm.nih.gov/.

Therapeutic strategies

The following therapeutic strategies are provided by way
25   of example only. The invention is not limited to a
theory of operation of a given antisense or siRNA.

Antisense

30   Herpes simplex viruses according to the present invention
may express an antisense nucleic acid, e.g. single
stranded RNA that targets and binds, by complementary

sequence binding, to the target mRNA thereby blocking translation of that mRNA and expression of the gene product.

5   Expressed antisense nucleic acid may also be arranged to bind sense genomic nucleic acid and inhibit transcription of a target nucleotide sequence.

siRNA

10

Herpes simplex viruses according to the present invention may encode nucleic acid designed such that on transcription an RNA having internal complementary sequence is provided and which may bind to form a short

15   hairpin siRNA duplex having a stem-loop structure. Preferably, the hairpin siRNA mediates specific repression and/or silencing of gene expression by RNA interference.

20   Alternatively, two siRNA molecules may be encoded which are designed to bind by complementary sequence binding and form a functionally active duplex molecule.

Repression and silencing

25

siRNA and antisense molecules provided under the invention are designed to repress or silence the expression of a target nucleic acid, peptide, polypeptide or protein or to repress a function of such nucleic acid,

30   peptide, polypeptide or protein.

A repression of expression results in a decrease in the
quantity or expressed function of the target. For
example, in a given cell the repression of SCCRO by
expression of an siRNA or antisense may result in a

5   decrease in either the quantity of the SCCRO gene product
or the expressed function of the SCCRO gene product
relative to an untreated cell.

Repression of a function may involve the decrease in

10   transcription of an mRNA, or translation of a peptide or
polypeptide.

Repression may be partial. Preferred degrees of
repression are at least 50%, more preferably one of at

15   least 60, 70, 80, 85 or 90%. A level of repression
between 90% and 100% is considered a 'silencing' of
expression or function.

Hybridisation stringency

20

In accordance with the present invention, nucleic acid
sequences may be identified by using hybridization and
washing conditions of appropriate stringency.

25   Complementary nucleic acid sequences will hybridise to
one another through Watson-Crick binding interactions.
Sequences which are not 100% complementary may also
hybridise but the strength of the hybridisation usually
decreases with the decrease in complementarity. The

30   strength of hybridisation can therefore be used to
distinguish the degree of complementarity of sequences
capable of binding to each other.

The "stringency" of a hybridization reaction can be readily determined by a person skilled in the art.

5   The stringency of a given reaction may depend upon factors such as probe length, washing temperature, and salt concentration. Higher temperatures are generally required for proper annealing of long probes, while shorter probes may be annealed at lower temperatures. The

10   higher the degree of desired complementarity between the probe and hybridisable sequence, the higher the relative temperature which can be used. As a result, it follows that higher relative temperatures would tend to make the reaction conditions more stringent, while lower

15   temperatures less so.

For example, hybridizations may be performed, according to the method of Sambrook et al., ("Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press,

20   1989) using a hybridization solution comprising: 5X SSC, 5X Denhardt's reagent, 0.5-1.0% SDS, 100 µg/ml denatured, fragmented salmon sperm DNA, 0.05% sodium pyrophosphate and up to 50% formamide. Hybridization is carried out at 37-42°C for at least six hours. Following hybridization,

25   filters are washed as follows: (1) 5 minutes at room temperature in 2X SSC and 1% SDS; (2) 15 minutes at room temperature in 2X SSC and 0.1% SDS; (3) 30 minutes-1 hour at 37°C in 1X SSC and 1% SDS; (4) 2 hours at 42-65°C in 1X SSC and 1% SDS, changing the solution every 30

30   minutes.

One common formula for calculating the stringency conditions required to achieve hybridization between nucleic acid molecules is to calculate the melting temperature T_m (Sambrook et al., 1989):

5

$$T_m = 81.5°C + 16.6Log \; [Na+] + 0.41(\% \; G+C) - 0.63 \; (\% \; formamide) - 600/n$$

where n is the number of bases in the oligonucleotide.

10

As an illustration of the above formula, using [Na+] = [0.368] and 50% formamide, with GC content of 42% and an average probe size of 200 bases, the T_m is 57°C. The T_m of a DNA duplex decreases by 1 - 1.5°C with every 1%

15 decrease in sequence complementarity.

Accordingly, nucleotide sequences can be categorised by an ability to hybridise to a target sequence under different hybridisation and washing stringency conditions

20 which can be selected by using the above equation. The T_m may be used to provide an indicator of the strength of the hybridisation.

The concept of distinguishing sequences based on the

25 stringency of the conditions is well understood by the person skilled in the art and may be readily applied.

Sequences exhibiting 95-100% sequence complementarity may be considered to hybridise under very high stringency

30 conditions, sequences exhibiting 85-95% complementarity may be considered to hybridise under high stringency conditions, sequences exhibiting 70-85% complementarity

may be considered to hybridise under intermediate
stringency conditions, sequences exhibiting 60-70%
complementarity may be considered to hybridise under low
stringency conditions and sequences exhibiting 50-60%%
5   complementarity may be considered to hybridise under very
low stringency conditions.

The invention includes the combination of the aspects and
preferred features described except where such a
10   combination is clearly impermissible or expressly
avoided.

Aspects and embodiments of the present invention will now
be illustrated, by way of example, with reference to the
15   accompanying figures. Further aspects and embodiments
will be apparent to those skilled in the art. All
documents mentioned in this text are incorporated herein
by reference.

20   **Brief Description of the Figures**

Figure 1. Generation of plasmid RL1.dIRES-GFP from
plasmids pNAT-IRES-GFP and RL1.del.

25   **Figure 2.** Agarose gel electrophoresis of *Hpa*I digested,
CIP treated, RL1.del. RL1.del was digested with *Hpa*I. The
digested DNA was then treated with Calf Intestinal
Phosphatase (CIP) to prevent the vector re-annealing to
itself in subsequent ligation reactions. A sample of the
30   digested/CIP treated DNA was electrophoresed, beside a
1Kbp DNA ladder (Promega), on a 1% agarose gel. *Hpa*I
linearises the vector at 8.6 Kbp.

Figure 3. Agarose gel electrophoresis of *NsiI/SspI*
digested pNAT-IRES-GFP (A) and purified/blunt-ended pCMV-
NAT-IRES-GFP-PolyA (B). Four *NsiI/SspI* digestions of

5   pNAT-IRES-GFP were electrophoresed, beside a 1Kbp DNA
Ladder (Promega) on a 1% agarose gel. The 5.4Kbp
fragments (pCMV-NAT-IRES-GFP-PolyA) were purified from
the gel. The purified DNA was blunt ended using Klenow
polymerase and a sample electrophoresed on an agarose gel

10   to check its concentration.

Figure 4. Identification of RL1.del clones containing the
pCMV-NAT-IRES-GFP-PolyA insert. Ligation reactions were
set up with the purified, blunt ended pCMV-NAT-IRES-GFP-

15   PolyA fragment and *HpaI* digested, CIP treated RL1.del.
Bacteria were transformed with samples from the ligation
reactions and plated out onto LBA (Amp^r) plates. Colonies
were picked and plasmid DNA was extracted and digested
with *AflII*. Digested samples were electrophoresed, beside

20   a 1Kbp DNA ladder (L) (Promega), on a 1% agarose gel.
*Clones 5 and 8 contained the pCMV-NAT-IRES-GFP-PolyA
insert as two fragments of the predicted size - 4.8Kbp
and 9.2Kbp - were generated from *AflII* digestion. Clones
without inserts would not be digested with *AflII* as there

25   is no *AflII* site in RL1.del.
N.B. Inserts could have been cloned in two orientations,
both of which were acceptable.

Figure 5. Determination of the orientation of pCMV-NAT-

30   IRES-GFP-PolyA in clone 5 (RL1.dCMV-NAT-GFPb). pCMV-NAT-
IRES-GFP-PolyA (blunt ended) could have been cloned into
the *HpaI* site of RL1.del in two orientations. To

determine the orientation of the insert in clone 5, the
plasmid was digested with *Xho*I and the digested DNA
electrophoresed, beside a 1Kbp DNA ladder (Promega), on a
1% agarose gel. If the insert had been cloned in the

5   orientation shown in A, two fragments of 10.2Kbp and
3.8Kbp would be generated from *Xho*I digestion. If it had
been cloned in the opposite orientation (B), two
fragments of 12.4Kbp and 1.6Kbp would be generated. The
presence of two fragments of 10.2Kbp and 3.8Kbp in the

10   gel confirmed that the insert had been cloned in the
orientation shown in A.
*This *Xho*I site was present in the initial cloning vector
(RL1.del), upstream of the *Hpa*I site into which pCMV-
NAT-IRES-GFP-PolyA was cloned.

15

Figure 6. Removal of pCMV-NAT from clone 5 (A) and large
scale plasmid preparation of RL1.dIRES-GFP (B). Four
samples of clone 5 were digested with *Xho*I and
electrophoresed, beside a 1Kbp DNA ladder (L) (Promega),

20   on a 1% agarose gel (A). The larger fragment of DNA
generated from this digestion (10.2Kbp) was purified from
the gel and ligated back together, at the *Xho*I sites, to
form a single *Xho*I site in a new plasmid, designated
RL1.dIRES-GFP. A large-scale plasmid preparation was

25   grown up and the preparation checked by digesting with
*Xho*I. 1µl and 4µl of the digested DNA was
electrophoresed, beside a 1Kbp DNA ladder (L) (Promega),
on a 1% agarose gel (B). The DNA should produce a single
fragment of 10.2Kbp when digested with *Xho*I. The *Cla*I,

30   *Bgl*II, *Nru*I and *Xho*I sites of RL1.dIRES-GFP are all
unique.

*Clone 5 is the RL1.del plasmid into which has been cloned the 5.4Kbp pCMV-NAT-IRES-GFP-PolyA fragment from pNAT-IRES-GFP.

5   **Figure 7.** Generation, detection and purification of ICP34.5 null HSV-1 expressing a gene product of interest.

Figure 8. Strategy used to clone pCMV-asSCCRO, from pUSEamp-asSCCRO, into RL1.dIRES-GFP. (1) Digest pUSEamp-

10 asSCCRO with *Ssp*1 and *Xho*I and purify the 1.96Kbp pCMV-asSCCRO fragment; (2) Digest RL1.dIRES-GFP with *Bgl*II, blunt end using Klenow polymerase and treat with Calf Intestinal Phosphatase (CIP). (3) Clone the blunt ended pCMV-asSCCRO fragment (1.96Kbp) into *Bgl*II digested/blunt

15 ended/CIP treated RL1.dIRES-GFP. (*pUSEamp-asSCCRO was provided by Memorial Sloan-Kettering Cancer Centre, New York.)

Figure 9. Agarose gel electrophoresis of *Bgl*II digested,

20 blunt ended, CIP treated RL1.dIRES-GFP. RL1.dIRES.GFP was digested with *Bgl*II. The digested plasmid was then blunt ended using Klenow polymerase and treated with Calf Intestinal Phosphatase (CIP) to prevent the vector re-annealing to itself in subsequent ligation reactions. A

25 sample of the digested/blunt ended/CIP treated DNA was electrophoresed, beside a 1Kbp DNA ladder (Promega), on a 1% agarose gel to check its concentration. pCMV-asSCCRO was subsequently cloned into this digested/CIP treated vector.

30

Figure 10. Agarose gel electrophoresis of *Ssp*I/*Xho*I digested pUSEamp-asSCCRO (A) and the purified pCMV-

asSCCRO fragment (B). Four samples of pUSEamp-asSCCRO
were digested with *Ssp*I and *Xho*I then electrophoresed,
beside a 1Kbp DNA ladder (L) (Promega), on a 1% agarose
gel. The 1.96Kbp fragments, consisting of DNA antisense
5   to the squamous cell carcinoma related oncogene (asSCCRO)
downstream of the CMV IE promoter (pCMV), were purified
from the gel, blunt ended using Klenow polymerase,
purified again and a sample of the purified DNA
electrophoresed on an agarose gel to check its
10 concentration.

Figure 11. Identification of RL1.dIRES-GFP clones
containing the pCMV-asSCCRO insert. Ligation reactions
were set up with the purified, blunt ended pCMV-asSCCRO
15 fragment and *Bgl*II digested, blunt ended, CIP treated
RL1.dIRES-GFP. Bacteria were transformed with samples
from the ligation reactions and plated onto LBA (Amp^r)
plates. Colonies were picked and plasmid DNA was
extracted and digested with *Bgl*II. Digested samples were
20 electrophoresed, beside a 1Kbp DNA ladder (L) (Promega),
on a 1% agarose gel.
*Clone 11 contained the pCMV-asSCCRO insert as two
fragments of the predicted size - 1.4Kbp and 10.8Kbp were
generated from *Bgl*II digestion. Clones without the insert
25 would not produce a fragment of 1.4Kbp when digested with
*Bgl*II.

Figure 12.   Determination of the orientation of pCMV-
asSCCRO in clone 11. The presence of an *Nru*I site, ~320bp
30 into the cloned pCMV-asSCCRO fragment, was utilized to
determine the orientation of pCMV-asSCCRO. Clone 11 was
digested with *Nru*I and electrophoresed, beside a 1Kbp DNA

44

ladder (L) (Promega), on a 1% agarose gel. If pCMV-
asSCCRO was in the desired orientation (A), *NruI*
digestion would produce a fragment of 1.64Kbp. If in the
opposite orientation (B), no 1.64Kbp fragment would be

5   generated from this digestion. The presence of a fragment
at 1.64Kbp in the gel confirmed that pCMV-asSCCRO was in
the desired orientation. (*This *NruI* site was already
present in the initial cloning vector (i.e. RL1.dIRES-
GFP)).

10

      Figure 13.   Agarose gel electrophoresis of *ScaI* digested
clone 11 (A) and HSV1716/CMV-asSCCRO/GFP virus titre (B).
Clone 11 (RL1.dCMV-asSCCRO-GFP) was digested with *ScaI*,
the digested DNA purified and 5µl electrophoresed, beside

15   a 1Kbp DNA ladder (Promega), on a 1% agarose gel, to
check its concentration. 80% confluent BHK cells were
then co-transfected with 10µl HSV17$^+$ DNA and an
appropriate volume of the remaining digested clone 11.
The cells were incubated at 37°C for 3 days until cpe was

20   evident. Recombinant viral plaques were picked under the
fluorescent microscope, purified and a virus stock, named
HSV1716/CMV-asSCCRO/GFP, grown up. HSV1716/CMV-
asSCCRO/GFP was titrated on BHK cells.

25   **Figure 14.** Cytotoxicity assay for cell lines SCC15 and
584 after infection with HSV1716 or HSV1716asSCCRO at MOI
of 1pfu/cell and 5pfu/cell.

      Figure 15. Cytotoxicity assay for cell lines 1483 and
30   1986 after infection with HSV1716 or HSV1716asSCCRO at
MOI of 1 pfu/cell and 5pfu/cell.

Figure 16. Cytotoxicity assay for cell line 1186 and 1386 after infection with HSV1716 or HSV1716asSCCRO at MOI of 1 pfu/cell and 5pfu/cell.

5   **Figure 17**. Viral proliferation assays for head and neck squamous cell carcinoma cell lines after infection with HSV1716 or HSV1716asSCCRO at MOI 1pfu/cell.

Figure 18. Infectivity assay- gfp expression 6 hours post
10   infection with 1716gfp virus.

Figure 19. Western blot results of the cell line SCC15 showing downregulation of SCCRO protein at 12 hours with HSV1716asSCCRO but not in 584.

15

Figure 20. Nude mice xenograft growth curves in SCC15 and 584 following single intratumoural injection of HSV1716 or HSV1716asSCCRO.

20   **Figure 21**. Nude mice xenograft growth curves in SCC15 following single intratumoural injection of PBS, HSV1716 or HSV1716asSCCRO.

Figure 22.

25

> (A)   SEQ ID No. 1 - a Human SCCRO nucleic acid
>   sequence. Also showing the amino acid sequence of
>   the encoded polypeptide (an SCCRO gene product);
> (B)   SEQ ID No. 2 - Amino acid sequence of the
30   polypeptide encoded by SEQ ID No.1;

(C)   SEQ ID No. 3 - a Human SCCRO nucleic acid
       sequence. Also showing the amino acid sequence of
       the encoded polypeptide (an SCCRO gene product);

(D)   SEQ ID No. 4 - Amino acid sequence of the
5   polypeptide encoded by SEQ ID No.3.

Figure 23.   (A) DNA nucleotide sequence encoding the
siRNA construct designed to target expression of the
SCCRO gene (SEQ ID No. 5); and (B) nucleotide sequence
10   encoding control siRNA (SEQ ID No 6). Sequences either
side of the central nucleotides are respectively
complimentary enabling the transcribed RNA to form a
hairpin structure (stem-loop) by binding of complementary
nucleotides.

15

Detailed Description of the Best Mode of the Invention

Specific details of the best mode contemplated by the
inventors for carrying out the invention are set forth
20   below, by way of example. It will be apparent to one
skilled in the art that the present invention may be
practiced without limitation to these specific details.

Vectors Useful for Generation of Herpes Simplex virus
25   Mutants

Mutant herpes simplex viruses of the invention may be
generated by use of nucleic acid vectors.

30   One such vector useful for generation of mutant herpes
simplex viruses according to the present invention is a

nucleic acid vector comprising, consisting or consisting
essentially of:
first and second nucleotide sequences corresponding to
nucleotide sequences flanking an insertion site in the
5   genome of a selected herpes simplex virus; and
a cassette located between said first and second
nucleotide sequences comprising nucleic acid encoding:
     a)   one or a plurality of insertion sites; and
     b)   a ribosome binding site; and
10   c)   a marker.

Another vector useful for generation of mutant herpes
simplex viruses according to the present invention is a
nucleic acid vector comprising, consisting or consisting
15   essentially of:
first and second nucleotide sequences corresponding to
nucleotide sequences flanking an insertion site in the
genome of a selected herpes simplex virus; and
a cassette located between said first and second
20   nucleotide sequences comprising nucleic acid encoding:
     a)   one or a plurality of insertion sites; and
     b)   a first regulatory nucleotide sequence; and
     c)   a marker.

25   The first and second nucleotide sequences may correspond
to nucleotide sequences flanking an insertion site formed
in, or comprising all or a part of, the ICP34.5 protein
coding sequence of the genome of a selected herpes
simplex virus.

30

The cassette may comprise a plurality of insertion sites,
each insertion site preferably formed by nucleic acid

encoding a specific restriction endonuclease site
('restriction site'). Together the restriction sites may
form a multiple cloning site (MCS) comprising a series of
overlapping or distinct restriction sites, preferably a
5   series of distinct restriction sites comprising one or
more of the ClaI, BglII, NruI, XhoI restriction sites.

The encoded components of the cassette may be arranged in
a predetermined order. In one arrangement, the one or
10   plurality of insertion sites is/are arranged upstream
(i.e. 5') of the ribosome binding site/first regulatory
sequence and the ribosome binding site/first regulatory
sequence is arranged upstream (i.e. 5') of the marker.

15   The first and second nucleotide sequences may comprise
nucleotide sequences having identity to regions of the
genome surrounding the insertion site in the selected
herpes simplex virus (the 'viral insertion site'). These
sequences enable the cassette to be incorporated at the
20   viral insertion site by homologous recombination between
the first and second nucleotide sequences and their
respective corresponding sequences in the viral genome.

Thus the first and second nucleotide sequences are
25   flanking sequences for homologous recombination with
corresponding sequences of a selected viral genome, such
homologous recombination resulting in insertion of the
cassette at the viral insertion site.

30   The first and second nucleotide sequences may correspond
to nucleotide sequences flanking an insertion site in the

RL1 locus of the HSV genome, more preferably in the
ICP34.5 protein coding sequence of the HSV genome.

The first and second nucleotide sequences may each be at
5   least 50bp in length, more preferably at least 100, 150,
200, 300, 400, 500, 600, 700, 800, 900, 1000, 1100, 1200,
1300, 1400, 1500, 1600, 1700, 1800, 1900 2000, 2100,
2200, 2300, 2400, 2500, 2600, 2700, 2800, 2900, 3000,
3100, 3200, 3300, 3400, 3500, 3600, 3700, 3800, 3900 or
10   4000bp in length. Each of the first and second
nucleotide sequences may have at least 50% sequence
identity to their corresponding sequence in the viral
genome, more preferably at least 60%, 70%, 75%, 80%, 85%,
90%, 92%, 94%, 95%, 96%, 97%, 98% 99% or 100% identity.
15   Identity of sequences is determined across the entire
length of a given nucleotide sequence. Where sequences
are of different length, sequence identity of the shorter
sequence is determined over the entire length of the
longer sequence.
20

The first and second nucleotide sequences may be
characterised by the ability of one strand of a given
sequence to hybridise with the corresponding single-
stranded complement of the HSV genome under varying
25   hybridisation stringency conditions. Suitably, the first
and second nucleotide sequences will hybridise with their
corresponding complement under very low, low or
intermediate stringency conditions, more preferably at
high or very high stringency conditions.
30

The viral insertion site is the position between the
genomic nucleotide sequences corresponding to the first

and second nucleotide sequences of the vector (the
'genomic' and 'vector flanking sequences' respectively)
at which homologous recombination will occur and may be
predetermined by selection of the vector flanking

5   sequences. Where the genomic flanking sequences are
immediately adjacent, the insertion site is the position
between the peripheral and immediately adjacent bases of
the two genomic flanking sequences, such that insertion
of the cassette separates the genomic flanking sequences.

10   Where the genomic flanking sequences are separated by one
or a plurality of bases in the viral genome, the
insertion site is formed by said one or a plurality of
bases which are excised from the genome by the homologous
recombination event.

15

The position of the viral insertion site may be
accurately selected by careful selection and construction
of the vector flanking sequences. Accordingly, the
vector may be constructed such that homologous insertion

20   of the cassette results in disruption of a chosen protein
coding sequence and inactivation of the respective gene
product or such that the cassette is inserted at a non-
protein coding region of the viral genome. The complete
genome sequences of several herpes simplex virus strains

25   have been reported and are publicly available. The
complete genome sequence for HSV-1 strain 17syn+ was
reported by Dolan et al[3] (incorporated herein by
reference) and the complete genome sequence of HSV-2
strain HG52 was reported by Dolan et al[4] (incorporated

30   herein by reference) and is available from the EMBL
database under accession code Z86099. Using this
information, the vector of the present invention may

preferably be designed for use in generating mutant HSV-1
(e.g. in strain 17 or F) or mutant HSV-2 (e.g. in strain
HG52).

5   The first and second nucleotide sequences (vector
flanking sequences) may each comprise sequence
corresponding to the RL terminal repeat region of the
genome of the selected HSV (e.g. HSV-1 strains 17 or F or
HSV-2 strain HG52). The vector flanking sequences may
10   comprise, consist or consist essentially of nucleotide
sequences of the RL repeat region which flank the ICP34.5
protein coding sequence. In flanking the ICP34.5 coding
sequence, one or both of the selected sequences may, in
the corresponding HSV genome, overlap, i.e. extend into,
15   the ICP34.5 protein coding sequence or one or both
sequences may be selected so as to not overlap the
ICP34.5 protein coding sequence. In a similar manner, the
selected sequences may be chosen to overlap completely or
partially other important encoded signals, e.g.
20   transcription initiation site, polyadenylation site,
defined promoters or enhancers. In this preferred
arrangement the insertion site will thus comprise all or
a part of the ICP34.5 protein coding sequence and/or be
such that the inserted cassette disrupts the ICP34.5
25   protein coding sequence.

The vectors described, comprising first and second
nucleotide sequences corresponding to regions of the RL
repeat region flanking and/or overlapping the ICP34.5
30   protein coding sequence, may be used in the generation of
ICP34.5 null mutants wherein all or a portion of the
ICP34.5 protein coding sequence is excised and replaced

52

during the homologous recombination event such that both
copies of the ICP34.5 coding sequence are disrupted.
The recombination may result in an insertion of nucleic
acid within the ICP34.5 protein coding sequence thereby

5   disrupting that sequence. In that case, successfully
transformed virus are thus mutants incapable of
generating the ICP34.5 active gene product from at least
one copy, and preferably from both copies, of the ICP34.5
gene.

10

Successfully transformed virus are thus mutants incapable
of generating the ICP34.5 active gene product.

Each component of the cassette may be positioned

15   substantially adjacent the neighbouring component such
that a single bicistronic transcript comprising or
consisting essentially of the mRNA encoding the
nucleotide sequence of interest, ribosome binding site
and marker is obtainable.

20

The vectors described may further comprise, consist, or
consist essentially of a nucleic acid encoding a
selectable marker such as a polypeptide or protein
conferring antibiotic resistance e.g. kanamycin

25   resistance or ampicillin resistance.

The vectors described are preferably DNA vectors,
particularly dsDNA vectors. The vector may be provided as
a linear or circular (plasmid) DNA vector. The vector

30   preferably contains nucleotide sequences, e.g.
restriction endonuclease site(s), permitting transition
between the two forms by use of DNA ligation and

restriction materials (e.g. enzymes) and techniques known
to the person skilled in the art. To achieve homologous
recombination with a selected HSV, the vector is
preferably provided in linear form.

5

One such vector provided by the inventors is plasmid
RL1.dIRES-GFP deposited in the name of Crusade
Laboratories Limited having an address at Department of
Neurology Southern General Hospital 1345 Govan Road Govan

10   Glasgow G51 5TF Scotland on 03 September 2003 at the
European Collection of Cell Cultures (ECACC), Health
Protection Agency, Porton Down, Salisbury, Wiltshire, SP4
0JG, United Kingdom under accession number 03090303 in
accordance with the provisions of the Budapest Treaty on

15   the International Recognition of the Deposit of
Microorganisms for the Purposes of Patent Procedure
(herein referred to as the 'Budapest Treaty').

RL1.dIRES-GFP provides a platform for generating a

20   plurality of 'shuttle vectors' which can exploit the
process of homologous recombination to transfer a
nucleotide sequence of interest (downstream of a selected
promoter) into the disabling RL1 locus of HSV-1,
generating easily identifiable, oncolytic, ICP34.5 null

25   HSV-1 mutants expressing the products of the nucleotide
sequence of interest, e.g. an RNA transcript or a
polypeptide, and GFP. RL1.dIRES-GFP thus provides for
ease of generation and purification of ICP34.5 null HSV.

30   RL1.dIRES-GFP is a useful vector for making second-
generation oncolytic viruses having enhanced cytotoxic
potential and which may express the product(s) of

54

selected gene(s) to enhance the oncolytic and/or
therapeutic effect of the administered virus.

The RL1.dIRES-GFP plasmid incorporates a multi-cloning

5   sequence (MCS), upstream of an internal ribosome entry
site (IRES), the GFP gene and SV40 polyadenylation
sequences flanked by HSV-1 RL1 sequences. Incorporation
of the encephalomyocarditis virus IRES (EMCV IRES)
permits translation of two open reading frames from a

10   single transcribed mRNA.

Following generation of a specific shuttle vector by
cloning of the nucleotide sequence of interest (and the
selected promoter) into RL1.dIRES-GFP, recombinant HSV-1

15   expressing the desired nucleic acid transcript or
protein, can be generated and purified within 2 weeks.
This compares with 2-3 months using prior art protocols.

In the ICP34.5 null HSV generated using the RL1.dIRES-GFP

20   plasmid provided by the inventors transcription of both
the nucleotide sequence of interest and GFP as a single
transcript is controlled by the same promoter upstream of
the nucleotide sequence of interest, the transcribed IRES
directing cap-independent translation of GFP. The

25   generated ICP34.5 null HSV are non-neurovirulent. By
modifying the RL1.dIRES-GFP plasmid to incorporate
appropriate flanking sequences surrounding the cassette
other gene-specific HSV null mutants expressing GFP can
be generated.

30

RL1.dIRES-GFP is promoterless, thus enabling a promoter
of choice to be incorporated in the homologously

recombined shuttle vector for controlling expression of
the nucleotide sequence of interest from the inserted
cassette.

5   Plasmid RL1.dIRES-GFP or modified plasmid shuttle vectors
thereof further comprising nucleotide sequence encoding a
nucleic acid transcript or polypeptide of interest may be
provided in isolated or purified form.

10   The vector may be a variant of plasmid RL1.dIRES-GFP.

As the plasmid RL1.dIRES-GFP is designed for tandem
expression of a sequence of interest and the marker gene
encoding green fluorescent protein (GFP). The sequence of
15   interest is cloned into RL1.dIRES-GFP along with its
promoter (e.g. CMV) such that the promoter drives
transcription of an mRNA for the sequence of interest
along with the IRES-GFP. Translation results in
expression of the GFP from the internal ribosomal entry
20   site and the gene of interest and promoter must be cloned
into RL1.dIRES-GFP in the correct orientation to achieve
this. There are a number of instances where this tandem
expression arrangement may be unsuitable and a variation
of the cassette design is favourable.
25
One example is the expression of siRNAs as short hairpin
RNAs using RNA polIII promoters such as H1 or U6. These
promoters are unable to drive the additional tandem
expression of the IRES-GFP as the RNApolIII expression
30   cassette is designed only to produce short transcripts.
Additionally, sequences of interest derived from genomic

DNA with strong mRNA shut-off signals in their 3'
untranslated regions may not support IRES-GFP expression.

5   Thus in some cases a cassette may be provided in which
the sequence of interest and marker are expressed
separately from independent promoters.

One variant contains a cassette in which the ribosome
binding site of plasmid RL1.dIRES-GFP is replaced with a
10   regulatory nucleotide sequence, preferably a strong,
constitutive promoter such as the Phosphoglycerokinase
promoter. The marker is thereby expressed under the
control of this (the 'first') regulatory sequence. The
nucleotide sequence of interest (e.g. antisense or siRNA)
15   is expressed under the control of a second regulatory
sequence upstream (5') of the nucleotide sequence of
interest, e.g. the CMV promoter. This vector variant is
particularly suitable for expression of siRNA where a
weak promoter may be used for expression of the siRNA
20   molecule or the nucleic acid encoding the siRNA may have
a strong termination signal making it difficult to
produce a single bi- or poly-cistronic transcript
containing the transcribed siRNA and marker sequence. In
this arrangement the transformed virus containing the
25   cassette integrated in the viral genome produces two
separate transcripts under the control of the first and
second promoters.

This cassette was constructed in the following manner.
30   The 1.3kbp blunt-ended EcoRI/AflII fragment that contains
the PGK promoter/GFP gene was obtained by restriction
digestion followed by Klenow treatment from the vector

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pSNRG and cloned into the RL1-del vector cut with the
restriction enzyme NruI that generates blunt ends.
Successful insertion of the PGK/GFP DNA was confirmed by
BamHI digestion and the orientation of the inserted DNA

5   identified using the unique XhoI site in RL1-del and the
BsrGI site at the 3' end of PGK/GFP. Plasmids with
PGK/GFP in both forward and reverse orientation were
obtained and the plasmids were designated RL1-dPGK/GFPfor
and RL1-dPGK/GFPrev. Expression of GFP was confirmed in

10   BHK cells transfected with the forward and reverse
orientation plasmids.

Thus, sequences of interest along with their own
promoters (although it is preferred that the PGK promoter

15   is not also used for this purpose) can then be cloned
into either RL1-dPGK/GFPfor or RL1-dPGK/GFPrev in either
orientation using the remaining unique BglII, XhoI or
HpaI unique restriction enzyme sites. The resulting
plasmid can be used to derive recombinant HSV in which

20   the marker GFP gene and the gene of interest are
expressed independently from their own promoters.

The vectors described may be constructed for use in
generating engineered HSV-1 or HSV-2 by insertion of a

25   nucleic acid cassette through a mechanism of homologous
recombination between nucleotide sequences flanking the
cassette and corresponding sequences in the selected
herpes simplex virus genome.

30   The vectors described may comprise and have use as:
        i)   gene delivery (gene therapy) vectors for delivery
                of a selected nucleotide sequence, e.g. antisense

nucleic acid or siRNA, to a specific locus of the
HSV genome; and/or

ii) expression vectors for expression of the delivered
nucleotide sequence of i) from the HSV genome
under the control of a selected regulatory
element; and/or

iii) vectors for the generation of HSV gene-specific
null mutants wherein the cassette is inserted at a
selected genomic location to disrupt the protein
coding sequence of a selected HSV gene such that
the gene product is inactive in the resultant
mutant virus.

The vectors described may be used in the manufacture of
engineered gene specific HSV null mutants, i.e. HSV
mutants incapable of expressing an active gene product of
a selected gene. They may be used in the manufacture of
engineered viruses which express a selected protein from
only one gene copy the other gene copy being disrupted or
modified such that it cannot express a functional gene
product. Such vectors may also be used in the manufacture
of a medicament, preferably comprising said gene specific
HSV null mutant, for use in treating cancer and tumours,
preferably by the oncolytic treatment of the tumour.

The vectors described may also be used in the manufacture
of engineered HSV mutants wherein the genome of the
mutant HSV comprises a nucleotide sequence which has been
inserted in the HSV genome by homologous recombination of
the cassette such that the nucleotide sequence is
arranged to be transcribed from the HSV genome under the
control of a regulatory element e.g. promoter, preferably

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a regulatory element forming part of the inserted
cassette, to produce an antisense transcript or siRNA.
Preferably the antisense nucleotide sequence is an
exogenous/ heterologous (i.e. non-HSV originating)

5   sequence. Such vectors may be used in the manufacture of
a medicament, preferably comprising the engineered HSV
mutant, for use in the treatment of disease, including
the oncolytic treatment of tumours.

10   The vectors described may also be used in the manufacture
of an engineered HSV mutant wherein the genome of the
mutant HSV comprises a nucleotide sequence which has been
inserted in a protein coding sequence of the HSV genome
by homologous recombination of the cassette such that the

15   mutant HSV is incapable of expressing the active gene
encoded by said protein coding sequence and wherein the
inserted nucleotide sequence is expressed under the
control of a regulatory element to produce an antisense
transcript or siRNA. Preferably, the regulatory element

20   forms part of the cassette. Such vectors may be used in
the manufacture of a medicament, preferably comprising
the engineered HSV mutant, for use in the treatment of
disease, including the oncolytic treatment of tumours.

25   The vectors described may be used to generate mutant HSV
by inserting the cassette into the genome of a selected
HSV, the method of generation may comprise providing a
vector described above, where the vector is a plasmid,
linearising the vector; and co-transfecting a cell

30   culture with the linearised vector and genomic DNA from
said HSV.

The co-transfection may be carried out under conditions effective for homologous recombination of said cassette into an insertion site of the viral genome.

5   The method may further comprise one or more of the steps of:

1)   screening said co-transfected cell culture to detect mutant HSV expressing said marker; and/or

2)   isolating said mutant HSV; and/or

10   3)   screening said mutant HSV for expression of the nucleotide sequence of interest or the RNA or polypeptide thereby encoded; and/or

4)   screening said mutant HSV for lack of an active gene product; and/or

15   5)   testing the oncolytic ability of said mutant HSV to kill tumour cells in vitro.

Example 1

20   **Construction of plasmid RL1.dIRES-GFP**

General Approach

Plasmid RL1.dIRES-GFP was generated in three stages,
25   illustrated in Figure 1.

1. The DNA sequences containing the CMV IE promoter (pCMV), the NAT gene, the internal ribosome entry site (IRES), the GFP reporter gene and the SV40
30   polyadenylation sequences were excised from pNAT-IRES-GFP using *Nsi*I and *Ssp*I and purified.

2. The purified pCMV-NAT-IRES-GFP-PolyA DNA fragment was cloned into RL1.del to form a new plasmid designated RL1.dCMV-NAT-GFP.

5   3. The pCMV-NAT DNA sequences of RL1.dCMV-NAT-GFP were excised using *Xho*I and the remainder of the plasmid re-ligated to form a novel plasmid designated RL1.dIRES-GFP. This novel plasmid contained a multi-cloning site (all sites shown are unique) upstream of an IRES, the GFP gene

10 and the SV40 polyA sequences all within the HSV-1 RL1 flanking sequences. Recombinant ICP34.5 null HSV-1, expressing a gene of interest in the RL1 locus, can be generated by cloning the gene of interest (downstream of a suitable promoter) into the multi-cloning site and co-

15 transfecting BHK cells with the plasmid and HSV-1 DNA. Recombinant virus expressing the target gene can be identified using GFP fluorescence.

Removal of the CMV promoter and noradrenaline transporter

20 gene (pCMV-NAT) from RL1.dCMV-NAT-GFP, followed by re-ligation of the remainder of the plasmid, resulted in a novel plasmid (RL1.dIRES-GFP) containing a multi-cloning site (MCS), upstream of the encephalomyocarditis virus internal ribosome entry site (EMCV IRES), the GFP

25 reporter gene and the SV40 PolyA sequences, all within RL1 flanking sequences. This novel arrangement of DNA sequences or 'smart cassette' allows ICP34.5 null HSV-1, expressing a gene of interest in the RL1 locus, to be easily generated by simply inserting the desired

30 transgene (downstream of a suitable promoter) into the MCS and co-transfecting BHK cells with the plasmid and HSV-1 DNA. The IRES situated between the GFP gene and the

MCS permits expression of two genes from the same
promoter and so recombinant virus expressing the gene of
interest also expresses GFP and can therefore be easily
identified under a fluorescence microscope and purified.

5

Materials and Methods

1μg of RL1.del* was digested with 10units *Hpa*I (Promega)
in a suitable volume of 10x buffer (Promega) and nuclease

10   free water (Promega) at 37°C for 16hrs. The digested
plasmid was then purified using the QIAquick PCR
purification kit (Qiagen), treated with 10 units of Calf
Intestinal Phosphatase (Promega), in a suitable volume of
10x CIP buffer and nuclease free water for 4hrs at 37°C,

15   before being purified again using a Qiaquick PCR
purification kit. 5μl of the purified DNA was
electrophoresed on a 1% agarose gel to check its
concentration (Figure 2).

20   4 x 1μg of pNAT-IRES-GFP** was digested with 10 units of
*Nsi*I and 10 units of *Ssp*I in a suitable volume of 10x
buffer (Promega) and nuclease free water (Promega) at 37°C
for 16hrs. The reaction mixture was electrophoresed in a
1% agarose gel for 1hr at 110 volts. The 5.4Kbp DNA

25   fragment consisting of the CMV IE promoter (pCMV),
upstream of the noradrenaline transporter gene (NAT), the
encephalomyocarditis virus internal ribosome entry site
(IRES), the gene for green fluorescent protein (GFP) and
the SV40 polyadenylation sequences (SV40 Poly A), was

30   excised using a sterile scalpel and the DNA purified from
the gel using a QIAquick Gel Extraction kit (Qiagen). The
eluted DNA was blunt ended using 3 units Klenow

Polymerase (Promega) in accordance with the manufacturers
instructions and the DNA purified using a QIAquick PCR
purification kit (Qiagen). 5µl of the purified DNA
fragment was electrophoresed on a 1% agarose gel to check
5   its concentration (Figure 3).

Ligation reactions were carried out in small eppendorf
tubes containing 5 units T4 DNA Ligase (Promega), a
suitable volume of 10X DNA Ligase Buffer (Promega),
10 nuclease free water (Promega) and various volumes of the
HpaI digested/CIP treated RL1.del and blunt ended pCMV-
NAT-IRES-GFP-SV40 Poly A DNA, at 16°C overnight. Competent
JM109 bacterial cells (Promega) were then transformed
with various aliqouts of the ligation reactions***.
15 Colonies formed on the plates were picked, had their
plasmid DNA extracted using a Qiagen Plasmid Mini kit and
screened for inserts using AflII (New England Biolabs)
restriction enzyme analysis. Plasmid DNA containing the
insert would produce two fragments of 4.8Kbp and 9.2Kbp
20 following digestion with AflII. Two clones (clone 5 and
8) contained the insert (Figure 4). The orientation of
the insert in clone 5 (RL1.dCMV-NAT-GFP) was determined
using XhoI restriction enzyme analysis (Figure 5).

25 To generate RL1.dIRES-GFP from clone 5, the CMV-NAT
portion of the CMV-NAT-IRES-GFP-SV40 PolyA insert was
removed by digesting 4 x 500ng of clone 5 with 10 units
of XhoI in a suitable volume of buffer and water
(Promega), overnight at 37°C. The digested DNA was
30 electrophoresed on a 1% agarose gel at 110 volts for 1hr
(Figure 6A). The 10.2Kbp fragment consisting of the IRES,
the GFP gene, the SV40 PolyA sequences and RL1 flanking

sequences in a pGEM3Zf(-) (Promega) backbone, was excised using a sterile scalpel and the DNA purified from the gel using a QIAquick Gel Extraction kit.

5   Ligation reactions were performed in small eppendorf tubes containing 100ng - 500ng purified DNA, 3 units T4 DNA Ligase (Promega), a suitable volume of 10X DNA Ligase Buffer (Promega) and nuclease free water (Promega) overnight at 16^0C. Competent JM109 bacterial cells
10 (Promega) were then transformed with various aliquots of the ligation reactions***. Colonies formed on the plates were picked, had their plasmid DNA extracted using a Qiagen Plasmid Mini kit and screened using *Xho*I (Promega) restriction enzyme analysis. Colonies containing plasmid
15 DNA from which CMV-NAT had been removed would produce one fragment of 10.2Kbp when digested with *Xho*I. Several positive clones were found, one was isolated, and a large-scale plasmid preparation undertaken using Promega's Wizard Plus Maxipreps kit. The large-scale
20 plasmid preparation was checked by digesting with *Xho*I (Figure 6B). This plasmid DNA was subsequently named 'RL1.dIRES-GFP'.

Plasmid RL1.dIRES-GFP has been deposited in the name of
25 Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 03 September 2003 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury,
30 Wiltshire, SP4 0JG, United Kingdom under accession number 03090303 in accordance with the provisions of the Budapest Treaty.

RL1.del

*RL1.del was provided by Dr.E.McKie and is the pGEM-3Zf(-) plasmid (Promega) into which has been cloned an HSV-1

5   fragment (123459-129403) consisting of the RL1 gene and its flanking sequences. The 477bp *Pfl*MI-*Bst*EII fragment of the RL1 gene (125292-125769) has been removed and replaced with a multi-cloning site (MCS) to form RL1.del.

10   *pNAT-IRES-GFP*

** pNAT-IRES-GFP was supplied by Dr. Marie Boyd (CRUK Beatson Laboratories) and is the pIRES2-EGFP plasmid (BD Biosciences Clontech) into which she has cloned the bovine noradrenaline transporter (NAT) gene (3.2Kbp), at

15   the *Nhe*I and *Xho*I sites.

****Transformation of Bacterial Cells*

10µl of a glycerol *E.coli* stock was added to 10ml 2YT medium in a 20ml griener tube. This was placed in a 37°C

20   shaking incubator for 16-24hrs until a saturated culture was obtained. 1ml of this culture was then added to 100ml of 2YT in a 500ml sterile glass bottle and placed in the 37°C shaking incubator for 3hrs. The bacterial cells were pelleted by centrifugation at 2,000rpm for 10

25   minutes (Beckman). The cells were then resuspended in 1/10th volume of transformation and storage buffer (10mM MgCl$_2$, 10mM Mg(SO)$_4$, 10% (w/v) PEG 3,500, 5% (v/v) DMSO). The cells were placed on ice for between 10 minutes and 2hrs, after which time they were considered competent for

30   transformation.

1-10μl of DNA was mixed with 100μl of competent bacteria
in eppendorf tubes, and the tubes placed on ice for 30
minutes. After this, the samples were 'heat shocked' by
incubating the tubes in a 42°C water bath for exactly 45

5   seconds before placing them on ice for a further 2
minutes. 1ml of L-Broth was added, the tube inverted 2-3
times, and the bacteria incubated for 1hr at 37°C. 100μl
of the transformed bacteria was plated out onto L-broth
agar plates containing 100μg/ml of the appropriate

10   antibiotic (usually ampicillin or kanamycin). Plates
were allowed to dry at room temperature, before
incubating in an inverted position at 37°C overnight.

Example 2

15

**Generation of ICP34.5 null HSV-1 expressing a gene
product of interest and GFP using plasmid RL1.dIRES-GFP.**

General Approach

20

Generation of ICP34.5 null HSV-1 expressing a gene
product of interest requires insertion of nucleotide
sequence encoding the gene product (polypeptide) of
interest and desired promoter at the MCS of RL1.dIRES.GFP

25   followed by co-transfection of BHK cells with the
linearised plasmid, containing the gene of interest, and
HSV DNA. Following homologous recombination viral plaques
expressing GFP are identified. Figure 7 illustrates the
method steps involved.

30

Referring to Figure 7A plasmid DNA, containing the gene
of interest and the desired promoter (X), is digested

with restriction endonucleases to release the
promoter/gene fragment.

The promoter/gene fragment is purified and cloned into
5   the multi-cloning site (MCS) of RL1.dIRES.GFP forming a
shuttle vector suitable for generating oncolytic HSV-1
(Figure 7B). This vector contains HSV-1 sequences that
flank the essential RL1 gene but does not contain the RL1
gene. The plasmid also contains the gene for Green
10   Fluorescent Protein (GFP) downstream of an internal
ribosome entry site (IRES). The IRES permits expression
of both the gene of interest and the GFP gene from the
same upstream promoter.

15   BHK cells are then co-transfected with linearised
RL1.dIRES.GFP, now containing the gene of interest, and
HSV-1 DNA (Figure 7C). Following homologous
recombination, designer virus, expressing the gene of
interest and GFP, is generated and can be distinguished
20   from wild type virus (also generated but not expressing
GFP) under a fluorescence microscope.

Viral plaques, expressing GFP (and hence the gene of
interest), are picked under the fluorescence microscope
25   and purified until all wild-type HSV-1 has been removed.
The recombinant HSV-1 is considered 100% pure when all
the viral plaques are expressing GFP (Figure 7D).

Once the recombinant virus is completely pure, an
30   isolated plaque is picked and a highly concentrated stock
is grown and titrated (Figure 7E). Oncolytic HSV-1,
expressing a gene product of interest from a selected

promoter, is then ready for characterisation and in vitro examination of its tumour killing potential.

Materials and Methods

5

To generate recombinant ICP34.5 null HSV-1 expressing a gene of interest and GFP, requires the gene of interest and a suitable promoter to be cloned into the MCS of RL1.dIRES-GFP in the forward orientation with respect to

10   the GFP gene in this plasmid. Once this has been achieved the plasmid is linearised (i.e. digested with a restriction enzyme that cuts only once, usually *SspI* or *ScaI*) in an irrelevant region. 80% confluent BHK cells in 60 mm petri dishes are then co-transfected with HSV-1 DNA

15   and linearised plasmid DNA as described below.

To generate replication restricted HSV-1, expressing the gene of interest and GFP, the gene of interest must be cloned into RL1dIRES-GFP downstream of a suitable

20   promoter (e.g. CMV IE). The promoter is required upstream of the gene of interest for the production of a bicistronic mRNA transcript. The IRES sequence between the two open reading frames in the transcript functions as a ribosome binding site for efficient cap-independent

25   internal initiation of translation. The design enables coupled transcription of both the gene of interest and GFP, followed by cap-dependent initiation of translation of the first gene (gene of interest) and IRES-directed, cap-independent translation of GFP. Co-ordinate gene

30   expression is thus ensured in this configuration.

Co-Transfection of Virus and Plasmid DNA by CaPO₄ and DMSO Boost

HSV-1 (17$^+$) DNA and 0.1-1µg linearized SMART cassette containing the gene and promoter of interest is pipetted

5   into 1.5ml eppendorf tubes containing 1µl of calf thymus DNA (10µg/ml) and an appropriate volume of distilled water to give a final volume of 165µl. The solutions are very gently mixed using a 200µl pipette tip. 388µl of HEBS, pH 7.5, (130mM NaCl, 4.9mM KCl, 1.6mM Na$_2$HPO$_4$, 5.5mM

10   D-glucose, 21mM HEPES) is then added, the solution mixed, before adding 26.5µl of 2M CaCl$_2$ dropwise and flicking the eppendorf tube two or three times. The samples are left at room temperature for 10-15 minutes then added dropwise to 80% confluent BHK's in 60mm petri dishes from which

15   the medium has been removed. Following incubation at 37°C for 45 minutes, the cells are overlaid with 5ml of ETC10 and incubated at 37°C. Three to four hours later, the media is removed and the plates washed with ETC10. For exactly 4 minutes, the cells are overlaid with 1ml 25%

20   (v/v) DMSO in HEBS at room temperature. After the 4 minutes, the cells are immediately washed three times with 5ml ETC10 before overlaying with 5ml of ETC10 and returning to the incubator. The following day, fresh medium is added to the cells. Two days later, when cpe

25   is evident, cells are scraped into the medium, transferred to small bijoux and sonicated thoroughly. The sample is then stored at -70°C until required (see section below on plaque purification).

30   **N.B.** The volume of virus DNA to add is determined by undertaking the above procedure without plasmid DNA,

using a range of virus DNA volumes and choosing the
volume that gives the greatest number of viral plaques on
the BHK monolayer after 2 or 3 days.

5   *Plaque Purification*
     Sonicated samples from co-transfection plates are thawed
     and serially diluted 10 fold in ETC10. 100μl from neat to
     the 10^5 dilution is plated out on confluent BHK's in 60 mm
     petri dishes from which the media has been removed.
10   After 45 minutes incubation at 37°C, the cells are
     overlaid with 5ml EMC10 and incubated at 37°C for 48hrs.
     The plates are then checked for the presence of viral
     plaques and those dishes with the fewest, most separated
     plaques are placed under a fluorescent stereomicroscope.
15   Recombinant virus, designed to express the green
     fluorescent protein (GFP) in addition to the gene of
     interest, can clearly be distinguished from wild type
     virus using a GFP filter. Fluorescent plaques are picked
     using a 20μl pipette and placed (including the tip) into
20   an eppendorf tube containing 1ml ETC10. The sample is
     thoroughly sonicated before making serial 10 fold
     dilutions in ETC10 and repeating the above purification
     procedure. The process is repeated typically 3-4 times
     until every plaque on the BHK monolayer is fluorescent.
25   Once this has been achieved, 50μl of this sample is used
     to infect BHK's in roller bottles, in 50ml ETC10, and a
     virus stock grown.

     Tissue Culture Media
30   BHK21/C13 cells are grown in Eagle's medium (Gibco)
     supplemented with 10% newborn calf serum (Gibco) and 10%
     (v/v) tryptose phosphate broth. This is referred to as

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ETC10. For virus titrations and plaque purification,
EMC10 (Eagles medium containing 1.5% methylcellulose and
10% newborn calf serum) is used to overlay the cells.

5   **Example 3**

Construction of HSV1716/CMV-asSCCRO/GFP

General Approach

10

HSV1716/CMV-asSCCRO/GFP was generated by first digesting
pUSEamp-asSCCRO with *Ssp*I and *Xho*I and purifying the
1.96Kbp fragment generated from the digestion. The
1.96kbp *Ssp*I/*Xho*I fragment comprises DNA antisense to

15   squamous cell carcinoma related antigen (asSCCRO),
downstream of the CMV IE promoter (pCMV). This fragment
was cloned into the MCS of the RL1.dIRES-GFP smart
cassette, in the forward orientation with respect to the
GFP gene in RL1.dIRES-GFP (Figure 8). The resultant

20   plasmid, named RL1.dCMV-asSCCRO-GFP, was then linearised
and recombinant virus generated and purified as described
in Example 2. The plasmid pUSEamp-asSCCRO was obtained
from Bhuvanesh Singh, Memorial Sloan Kettering Cancer
Center, New York.

25

Materials and Methods

2µg of the RL1.dIRES-GFP plasmid was then digested with
15 units of *Bgl*II (Promega), in a suitable volume of 10x

30   buffer (Promega) and nuclease free water (Promega), at
37°C for 16hrs. The digested plasmid was then purified
using the QIAquick PCR purification kit (Qiagen), treated

with 5 units of Klenow polymerase (Promega) for 20
minutes at room temperature, then purified again. The
purified DNA was then added to 10 units of Calf
Intestinal Phosphatase (Promega), in a suitable volume of
5   10x CIP buffer and nuclease free water for 4hrs at 37°C,
before being purified again using the QIAquick PCR
purification kit. 5µl of the purified DNA was
electrophoresed on a 1% agarose gel to check its
concentration (Figure 9).

10

4 x 1µg of pUSEamp-asSCCRO was digested with 10 units of
SspI and 10 units of XhoI (Promega), in a suitable volume
of 10x buffer (Promega) and nuclease free water
(Promega), at 37°C for 16hrs. The reaction mixture was
15   electrophoresed in a 1% agarose gel for 1hr at 110 volts.
The 1.96Kbp DNA fragment, consisting essentially of the
CMV promoter upstream of DNA antisense to SCCRO (pCMV-
asSCCRO), was excised using a sterile scalpel and the DNA
purified from the gel using a QIAquick Gel Extraction kit
20   (Qiagen). The purified DNA was blunt ended using 5 units
of Klenow polymerase (Promega) for 20 minutes at room
temperature, then purified again. 5µl of the purified DNA
fragment was electrophoresed on a 1% agarose gel to check
its concentration (Figure 10).

25

Ligation reactions were carried out in small eppendorf
tubes containing 5 units T4 DNA Ligase (Promega), a
suitable volume of 10X DNA Ligase Buffer (Promega),
nuclease free water (Promega) and various volumes of the
30   BglII digested/blunt ended/CIP treated RL1.dIRES-GFP
plasmid and blunt ended pCMV-asSCCRO, at 16°C overnight.
Competent JM109 bacterial cells (Promega) were then

transformed with various aliqouts of the ligation
reactions. Colonies formed on the plates were picked, had
their plasmid DNA extracted using a Qiagen Plasmid Mini
kit and screened for inserts using *Bgl*II (Promega)

5   restriction enzyme analysis. RL1.dIRES-GFP plasmid DNA
containing the pCMV-asSCCRO insert would produce two
fragments of 10.8Kbp and 1.4Kbp following digestion with
*Bgl*II. One clone (clone 11) was found to contain the
insert (Figure 11). The pCMV-asSCCRO insert could have

10   been cloned into RL1.dIRES-GFP in two orientations. To
confirm that the pCMV-asSCCRO fragment had been cloned
into RL1.dIRES-GFP in the desired orientation, clone 11
was digested with 10 units of *Nru*I (Promega), in a
suitable volume of 10x buffer (Promega) and nuclease free

15   water (Promega), at 37°C for 16hrs. If the insert was in
the correct orientation, a fragment of 1.64Kbp would be
generated. As a 1.64Kbp fragment was generated following
digestion with *Nru*I (Figure 12), it was confirmed that
pCMV-asSCCRO had been cloned in the desired orientation.

20   This plasmid (clone 11) was named 'RL1.dCMV-asSCCRO-GFP'.

0.1-1µg of RL1.dCMV-asSCCRO-GFP was linearized by
digesting with 10 units of *Sca*I (Promega), in a suitable
volume of 10x buffer (Promega) and nuclease free water

25   (Promega), at 37°C for 16hrs. A sample (5µl) of the
digested DNA was electrophoresed on a 1% agarose gel for
1hr at 110 volts to check that it had been linearized.
80% confluent BHK cells were then co-transfected with a
suitable volume of the remaining linearised DNA and HSV-1

30   DNA. Recombinant HSV-1, expressing GFP (and hence
asSCCRO), was identified and purified using a fluorescent
microscope and a virus stock, named HSV1716/CMV-

asSCCRO/GFP, was grown and titrated on BHK cells (Figure 13).

HSV1716/CMV-asSCCRO/GFP has been deposited as 'HSV1716asSCCRO' in the name of Crusade Laboratories Limited having an address at Department of Neurology Southern General Hospital 1345 Govan Road Govan Glasgow G51 5TF Scotland on 19 May 2004 at the European Collection of Cell Cultures (ECACC), Health Protection Agency, Porton Down, Salisbury, Wiltshire, SP4 0JG, United Kingdom under accession number 04051901 in accordance with the provisions of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure (herein referred to as the 'Budapest Treaty').

Example 4

The use of HSV1716asSCCRO as a novel therapeutic agent for head and neck squamous cell cancer.

The inventors believe that insertion of the antisense to SCCRO into the herpes simplex virus HSV1716 may provide a virus with a dual hit mechanism of cell kill. This would involve virus induced cell death via cytolysis in addition to cell death via downregulation of endogenous SCCRO expression.

The HSV1716asSCCRO virus was constructed, amplified and purified in accordance with the present invention. Following this, in vitro and in vivo analysis was carried out on a series of head and neck squamous cell cancer

(HNSCC) cell lines. HNSCC cell lines studied were SCC15,
1483, 1186, 1386, 1986 and 584. The relative expression
of SCCRO protein expression in these cell lines was
initially determined by western blotting. This showed the

5   cell lines SCC15, 1483 and 1186 had high levels of
expression of SCCRO, 1386 intermediate expression and
1986, 584 low expression. All cell lines were then
infected with HSV1716 or HSV1716asSCCRO viruses and
cytotoxicity determined by LDH release cytotoxicity assay

10   at MOI (multiplicity of infection) of 1 and 5pfu/cell
(Figures 14, 15 and 16). Viral proliferation was
determined by serial plaque assays at an MOI of 1pfu/cell
(Figure 17) and infectivity determined by green
fluorescent protein (gfp) using HSV1716gtp virus (Figure

15   18). In the cell lines with low or intermediate
expression (1986,584,1386) cytotoxicity increased in a
dose dependent fashion with both viruses but there was no
significant difference in cytotoxicity between the 2
viruses. Viral proliferation assays (Figure 17) showed

20   an increase in viral production over a range of 10^2 to 10^4
with equivalent proliferation with both viruses. In the
cell lines with high expression of SCCRO the inventors
found that the cell line SCC15 showed enhanced
cytotoxicity with the HSV1716asSCCRO virus. This

25   observation occurred 12 hours post viral infection which
is premature for virus induced cell death by a cytolytic
mechanism. In addition, virus proliferation of the 2
viruses was equivalent with an increase in virus
production of 10^4 for both viruses. These results

30   suggested that the enhanced cell kill at 12 hours was by
an alternative mechanism possibly by downregulation of
the endogenous high expression of SCCRO by antisenseSCCRO

expression. To investigate this hypothesis the inventors
analysed the cell lines SCC15 (high expression) and 584
(low expression) post virus infection by serial protein
expression over a 36 hour period. Cells were infected at

5   an MOI of 1pfu/cell with HSV1716 or HSV1716asSCCRO and
cells harvested and lysed for protein at 12, 24 and 36
hours post infection. Western blotting of the cell line
SCC15 showed downregulation of SCCRO protein at 12 hours
with HSV1716asSCCRO but not in 584 (see Figure 19). This

10   suggested that this was the mechanism by which
HSV1716asSCCRO had enhanced efficacy in cell line SCC15.

In vivo studies were then carried out in the cell lines
SCC15 and 584. Subcutaneous tumour were grown in athymic

15   nude mice and injected intratumorally with a single
injection of HSV1716, HSV1716asSCCRO or PBS control and
tumour sizes monitored at serial time points (Figure 20
and 21). In SCC15, efficacy was enhanced with
HSV1716asSCCRO compared to HSV1716. All 6 mice injected

20   with HSV1716asSCCRO showed complete responses by 21 days
post infection. Inhibition of tumour growth occurred with
HSV1716 with only 3/6 mice showing a complete response
over a 48 day follow up period. In the cell line 584,
both viruses were able to inhibit tumour growth but

25   neither virus produced a complete response in any mouse
xenograft injected. This in vivo data was further
evidence that HSV1716asSCCRO was a more potent antitumour
agent than HSV1716 in the cell line SCC15 with high SCCRO
expression.

30

These results suggest that HSV1716 and HSV1716asSCCRO has
great potential as useful therapeutic agents in the

treatment of recurrent or locally advanced head and neck
cancer by direct intratumoral injection. However, this
data also suggests that HSV1716asSCCRO may augment anti-
tumour activity in SCCRO over-expressing tumours. Since

5   SCCRO is overexpressed in a significant number of
squamous cell cancers of the head and neck this modified
virus may be particularly efficacious in this disease.
Therefore, the inventors believe that HSV1716asSCCRO will
be an important therapeutic agent in head and neck cancer

10   patients with locally advanced or recurrent head and neck
cancer, particularly as these cancers are amenable to
direct intratumoural injection.

Example 5 - Construction of HSV1716 variants expressing

15   **siRNA**

General Strategy

A plasmid that contains the siRNA construct designed to

20   target expression of the SCCRO·gene (SEQ ID No. 5) and
designated 339i was provided by Dr Bhuv Singh, MSKCC, New
York. A plasmid encoding a control siRNA (SEQ ID No 6),
designated Coni, was also provided.

25   Both siRNA constructs were in the vector pSNRG and their
expression is driven by the RNA polIII H1 promoter. RNA
polIII only transcribes short RNA molecules and the H1
promoter would be insufficient to drive expression of
IRES-gfp from the normal recombinant virus producing

30   shuttle vector RL1-del.IRES.gfp so an alternative cloning
strategy was adopted.

78

A cassette was constructed in the following manner. The
1.3kbp blunt-ended EcoRI/AflII fragment that contains the
PGK promoter/GFP gene was obtained by restriction
digestion followed by Klenow treatment from the vector
5   pSNRG and cloned into the RL1-del vector cut with the
restriction enzyme NruI that generates blunt ends.
Successful insertion of the PGK/GFP DNA was confirmed by
BamHI digestion and the orientation of the inserted DNA
identified using the unique XhoI site in RL1-del and the
10   BsrGI site at the 3' end of PGK/GFP. Plasmids with
PGK/GFP in both forward and reverse orientation were
obtained and the plasmids were designated RL1-dPGK/GFPfor
and RL1-dPGK/GFPrev. Expression of GFP was confirmed in
BHK cells transfected with the forward and reverse
15   orientation plasmids.

Thus, sequences of interest along with their own
promoters (in this arrangement it is preferred that a
different promoter is used to drive transcription of the
20   nucleotide sequence of interest and marker) can then be
cloned into either RL1-dPGK/GFPfor or RL1-dPGK/GFPrev in
either orientation using the remaining unique BglII, XhoI
or HpaI unique restriction enzyme sites. The resulting
plasmid can be used to derive recombinant HSV in which
25   the marker GFP gene and the gene of interest are
expressed independently from their own promoters

Materials and methods

30

In the pSNRG plasmid and adjacent to the H1/siRNA coding
sequence is a green fluorescent protein (gfp) expression
cassette   comprising   the   gfp   gene   with   a

Phosphoglycerokinase   (PGK)   promoter.   Using   the
restriction enzymes HindIII and AflII sequentially, the
1.6kbp DNA fragment that contains the H1/siRNA and
PGK/EGFP expression cassettes were excised from their

5   Coni and 339i plasmids. The 1.6kbp DNA fragment was
purified from a 1% agarose gel and blunt-ended by
incubation with Klenow DNA polymerase for 30 minutes at
30°C. The blunt-ended fragment was ligated into the RL1-
del shuttle vector which had been digested with the

10   restriction enzyme Nru1 that produces a blunt-ended cut.
Before ligation the Nru1-cut RL1-del was gel purified and
phosphatase-treated   using   Calf   Intestinal   Alkaline
Phosphatase. After an overnight ligation with either the
blunt-ended 339i or Coni DNA fragments with the blunt-

15   ended RL1-del plasmid, the reaction mix was used to
transform DH5alpha cells and these were plated-out on LB
amp plates. After overnight incubation at 37°C, individual
clones from each of the LB amp plates were grown
overnight in 3ml of LB broth and plasmid DNA extracted.

20

To screen for recombinants, plasmids were initially
digested with BamHI, as insertion of the H1/siRNA and
PGK/gfp cassette increases the size of the RL1 BamHI
fragment in the plasmid from 5.4kbp to 7.0kbp. For both

25   Coni and 339i ligations 1/24 clones screened demonstrated
a 7.0kbp BamHI fragment and the presence of the H1/siRNA
and PGK/EGFP cassette in these plasmids was confirmed by
EcoR1, EcoR1/HindIII and EcoR1/SalI digests, the inserted
H1/siRNA and PGK/EGFP cassette introduces a novel EcoR1

30   site into the RL1-del vector.

From a glycerol stock of the positive 339i and Coni clones, additional plasmid was prepared and used to transfect BHK cells. Fifty microlitres (50µl) of plasmid was mixed with 6µl lipofectamine 2000 in a final volume

5   of 100µl serum free medium and used to transfect BHK cells plated out on a 13mm glass coverslip in a 24-well plate. After 48hrs of transfection the cells were washed once in PBS, incubated for 2hrs in 4% paraformaldehyde, washed once more in PBS and mounted on microscope slides

10   using Vectashield. The presence of c5% gfp-positive cells following transfection with the RL1-del/339i and RL1-del/Coni plasmids confirmed the presence of the PGK/GFP cassette.

15   The RL1-del/339i and RL1-del/Coni plasmids were linearized using either of the restriction enzymes ScaI and XmnI and the linearized plasmid was used along with viral DNA to transfect BHK cells plated out to c80% confluency in 60mm dishes. To 100µl of linearized plasmid

20   or undigested circular plasmid, 50µl of HSV-1 strain 17+ DNA was added along with 20µl lipofectamine 2000 in a final volume of 500µl serum free medium and the mix added to the BHK cells. After 4hrs of transfection, the cells were shocked with 25% DMSO in HBSS for exactly 4 minutes,

25   washed x3 with medium and returned to 37°C incubation in 5ml of medium for 48hrs. Viral cpe was evident after 48hrs and the cells and medium were harvested together, sonicated and stored at -80°C. Undiluted medium/cells and 4x 10-fold dilutions were plated out on BHK cells and,

30   after 48hrs, viral plaques were examined by fluorescent microscopy for gfp expression. On the undiluted plate

from cells transfected with XmnI-linearized plasmid >100
gfp-positive plaques were observed for both Coni and 339i
indicating a high degree of recombination. Interestingly,
recombination,   but   at   a   lower   frequency   (c50

5   plaques/plate), was observed for the transfected circular
plasmid   but   recombination   with   the   ScaI-linearized
plasmid was very low (<5 plaques/plate).

Using the highest dilution at which gfp-positive plaques

10   were   clearly   visible   (the   PGK/GFP   cassette   gave   a   very
strong   fluorescent   signal),   two   plaques   each   of   Coni   and
339i   viruses   were   picked   using   a   sterile   pipette   tip,
placed   in   1ml   medium,   sonicated   for   1   minute   and   stored
at   -80°C.   Plaques   were   then   subjected   to   6   rounds   of

15   plaque   purification,   after   the   6th   round   no   wild   type,
non-gfp   expressing   plaques   were   visible   and   6   plaques
each   of   Coni   or   339i   virus   were   picked   for   Southern
blotting.

20   Each   of   the   six   plaques   of   Coni   and   339i   virus   was   used
to   infect   a   T175   flask   of   Vero   cells,   after   72hrs   of
infection   virus   was   harvested   and   titred.   For   3   each   of
the   Coni   and   339i   viruses   that   gave   the   highest   titres
0.5ml   was   used   to   infect   a   second   T175   flask   for   24hrs.

25   Viral   DNA   was   then   harvested   from   each   of   the   6   flasks.
The   BamHI-digested   viral   DNA   was   Southern   blotted   with
the   Alu/Rsa   RL1   probe   and   the   band   pattern   compared   to
wild   type   and   HSV1716   DNA   digested   also   with   BamHI.   A
novel   c6kbp   band,   consistent   with   the   insertion   of   the

30   1.6kbp   H1/siRNA   and   PGK/GFP   cassette   in   the   RL1   locus,
was   clearly   visible   in   all   six   viral   isolates   and   no   wild
type   bands   were   detected.   Stocks   of   the   Coni   and   339i

viruses that gave the strongest signal on Southern blotting were produced.

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